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13. ABSTRACT (Maximum 200) In order to determine whether differences exist in expression of estrogen receptor (ER) variant mRNAs between normal and tumor breast tissues, we have analyzed by reverse transcription-polymerase chain reaction (RT-PCR) ER variants mRNA expression in matched normal breast tissue and breast tumor components. A higher expression of exon 5-deleted, and of ERC4 variants in the tumor component compared to the normal counterpart of matched samples was seen whereas a higher expression of exon 3-deleted ER variant in the normal tissue was observed. This is consistent with previous observations made on independent samples. We have described the presence within normal breast as well as in breast tumor tissues of several variant forms of ER- β mRNA deleted in exon 5, exon 6, exon 5+6 or exon 8 sequences. The balance between some of these different ER- β isoforms was modified during breast tumorigenesis and tumor progression. We also showed that a higher ER- α /ER- β ratio was observed in the breast tumors compared with their matched normal breast tissues. This increase was attributed to an increase in ER- α mRNA expression and a lower ER- β mRNA expression in the tumor compared with that of the normal component in some ER+ cases. In addition, we showed that the expression of the newly described steroid receptor RNA activator SRA was also increased in breast tumor tissue and that the expression of a deleted SRA isoform, function of which remains to be determined, correlates with tumor grade. We concluded that ER signaling pathway involves a complex population of ER- α and ER- β like molecules and that the balance between their respective expression, that likely modulates the action of estrogens as well as antiestrogens, is modified during breast tumorigenesis and tumor progression.				
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FOREWORD

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INTRODUCTION

Estrogen is an important regulator of normal breast tissue growth and tumor development.¹ When this proposal was submitted, estrogens were thought to act mainly through a single receptor, now referred to as ER- α . ER- α is a ligand-activated transcription factor that belongs to the steroid/retinoic acid/thyroid receptor super family.² ER- α mRNA contains 8 different exons encoding a protein divided into structural and functional domains (A-F) (see Figure 1).³

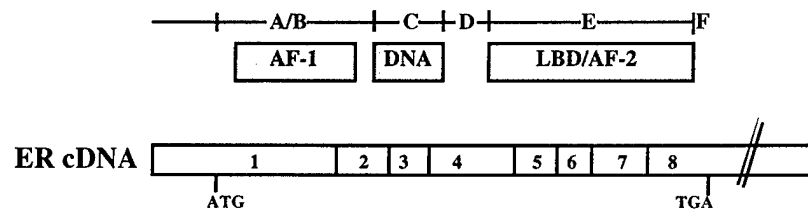
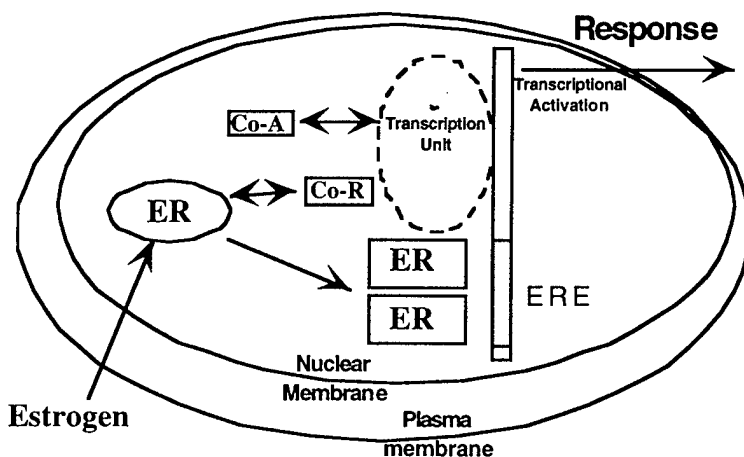


Figure 1: Schematic representation of ER- α cDNA and of the functional domain of the corresponding protein

Region A/B of the receptor is implicated in trans-activating function 1 (AF-1). The DNA-binding domain (DBD) is located in the C region. Region E is implicated in hormone binding (ligand binding domain, LBD) and another trans-activating function (AF-2). The mechanisms by which steroid receptors modulate the transcription of target genes have been extensively studied (for a review see McKenna et al.).⁴ Once bound to the ligand, the receptors undergo conformational changes and dimers of receptors recognize specific estrogen responsive elements (ERE) located on the DNA upstream of the target genes (See Figure 2).



Activated receptors, through interactions with co-activator proteins (Co-A), direct the assembly and the stabilization of a preinitiation complex that will ultimately conduct the transcription of these genes (see ref. 4 and references herein). Interactions with co-repressors (Co-R) will inhibit this action.

Figure 2: Schematic representation of ER- α mechanism of action

Several ER- α variant mRNAs have been described, that are missing one or more of the exons contained in the wild-type molecule (WT-ER- α). For a review of the different ER- α variants and their putative functions, see APPENDIX 1.⁵ The putative encoded proteins lack some of the WT-ER- α structural domains. Some of these proteins, such as those encoded by exon-3- deleted (ERD3), exon-7-deleted (ERD7) and exon-5-deleted (ERD5) ER- α variant mRNAs have been shown *in vitro* to interfere with WT-ER- α signal transduction. Indeed, ERD3 variant protein, that is missing the second zinc finger of the DNA binding domain, has been shown to have a dominant negative activity on WT-ER- α receptor action.⁶ Similarly, ERD7 variant protein, that is missing a part of the hormone binding domain, also acted as a dominant negative regulator of WT-ER- α .⁷ Interestingly, ERD5 variant protein, that is missing the hormone binding domain and the AF-2 region, displays a constitutive hormone independent activity and a WT-ER- α enhancing activity in different systems.^{8, 9} One should note that the activity of these different ER- α variants was found to be cell and promoter specific.

The ability of ER- α variants to interfere with WT-ER- α signaling pathway raised the hypothesis of their possible involvement in the acquisition of estrogen-independence that occurs during breast tumor progression. This hypothesis is supported by the observation of different levels of expression of ER- α variants in tumors at different stages of tumor progression. Indeed, ERD5 mRNA expression relative to WT-ER- α is higher in ER-/PR+ than in ER+/PR+ tumors.¹⁰ More recently, Gallacchi et al. reported that an increased expression of ERD5 variant mRNA levels was observed in tumors relapsing within 15 months compared to tumors not relapsing within the same amount of time.¹¹ The expression of ERD7 mRNA variant was shown to be higher in ER+/PR- than in ER+/PR+ tumors.⁷ Similarly, relatively higher levels of ERC4 variant mRNA, a variant truncated after sequences encoding exon 2 of the wild-type ER- α mRNA, were found in tumors with markers of poor prognosis and lack of hormone sensitivity compared to those with markers of good prognosis and hormone sensitivity.¹² Taken together, these data suggest that changes in the relative expression of particular ER- α variants occur during breast tumor progression.

We have previously demonstrated that ER- α variant mRNAs were expressed in normal human breast and that the expression of some of these variants was lower than that observed in independent breast tumor samples. Indeed, the expression of ERD5 and ERC4 variant mRNAs relative to WT-ER- α , was significantly increased in a group of breast tumors compared to unmatched normal reduction mammoplasty samples.^{13,14} Such data suggest that the molecular mechanisms generating ER variant mRNAs pre-exist in normal breast and could be deregulated in breast cancer tissues. We hypothesized that the deregulation of these mechanisms and therefore of ER signaling pathway may contribute to early steps in breast tumorigenesis.

The initial goal of this project was to address the possible role of Estrogen Receptor variants in human breast tumorigenesis. The initial objectives were:

1. *To look for differences in the expression of already described forms of ER- α variant mRNAs between matched normal breast tissue, invasive primary carcinoma, and metastatic carcinoma in axillary lymph nodes.*
2. *To identify variant ER mRNAs differentially expressed in normal breast and breast cancer tissue.*
3. *To determine the putative function of differentially expressed variants.*

To reduce the possible impact of patient variability, this study was performed using matched tissues (normal breast tissue, invasive primary carcinoma, and metastatic carcinoma in axillary lymph nodes) samples coming from the same patients.

Since the award of this grant, several important papers have been published that led us to re-evaluate this project and to establish additional objectives.

In 1996, Deng et al. reported differences between morphologically "normal" tissue adjacent to a tumor component versus "real" normal tissue geographically isolated from the tumor component.¹⁵ In the general context of our study it became important to check whether the normal breast tissue of women **with** breast cancer differed from the normal breast tissue of women **without** breast cancer. An additional objective was therefore established.

4. *Does the expression of particular ER- α variant mRNAs differ between the normal breast tissue of women with and without diagnosed breast cancer ?*

The recent cloning of a new estrogen receptor,¹⁶ now referred to as ER- β , is leading to a total re-evaluation of estrogen signal transduction in target tissues. This receptor shares the same structural and functional composition as the ER- α and has strong sequence similarities within the DNA binding domain and the ligand binding with ER- α (Figure 3).

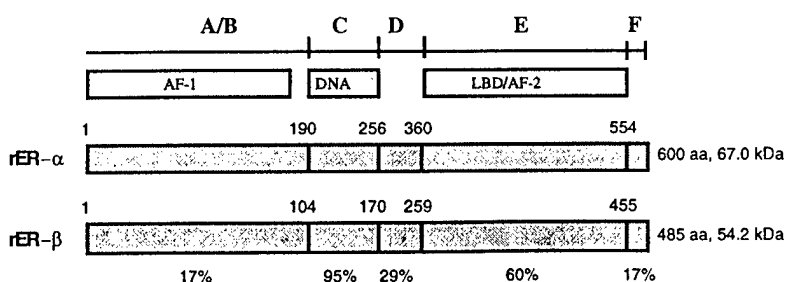


Figure 3: Structural and functional domains of rat ER- α and rat short ER- β : For each receptor, the length (aa), the calculated mass (kDa) and the amino acid positions of the different domains are given. Percentage amino acid identity in each domain is indicated.

This new receptor is able to form heterodimers with ER- α and data have been accumulating which suggest cross-talk between ER- β and ER- α signaling pathways. For a review of ER- β characteristics, see **APPENDIX 2**.¹⁷ It therefore appeared that the action of estrogen had to be considered in the context of the two signaling pathways.

This observation led us to include additional objectives in our project.

5. *Is ER- β expressed in human breast tumors ?*
6. *What is the relationship between ER- β expression and steroid receptor status in breast tumors ?*
7. *Do equivalent ER- β variants exist ?*
8. *Is the relative expression of these variants modified during breast cancer progression and tumorigenesis ?*
9. *Does the balance between ER- α and ER- β change during breast tumorigenesis ?*

Lanz et al. recently added to the already long list of estrogen receptor co-activators, the steroid receptor RNA activator (SRA).¹⁸ SRA differs from other co-activators in two main features. Firstly, SRA transcripts do not appear to be translated and therefore, this co-activator acts as an RNA and not as a protein. Lanz et al. showed that SRA exists in a ribo-nucleoprotein complex that contains SRC-1 and is recruited by steroid receptors. Secondly, SRA appears to be actually specific for steroid receptors. Indeed, most of the receptor-interacting factors, such as SRC-1 or TIF2/hSRC-2, interact with and co-activate both class I and class II nuclear receptors (see ref. 4 and references herein). Of particular interest is the fact that SRA interacts with the activation function 1 (AF-1) of the steroid receptors. AF-1, that is thought to mediate the agonistic effect of antiestrogens such hydroxytamoxifen¹⁹ is indeed the only domain that is conserved in all ER- α variants described to date.

Two additional objectives were therefore included in this study.

10. *Is SRA expressed in breast tumor tissues ? If yes, is this expression changed during breast tumor progression?*
11. *Is SRA expressed in normal breast tissues ? If yes, do changes occur in the level of SRA expression during breast tumorigenesis.*

BODY

*Objective 1: Comparison of the relative expression ERC4, ERD3 and ERD5 variant mRNAs in matched adjacent normal and tumorous breast tissues (APPENDIX 3).*²⁰

Eighteen cases were selected from the Manitoba Tumor Data Bank files from which matched normal breast tissue and primary invasive carcinoma were available. Among these cases, 6 were ER-/PR- (i.e ER levels lower than 3 fmol/mg protein and PR levels lower than 10 fmol/mg, as determined by ligand binding assay), 1 is ER-/PR+, 2 are ER+/PR-, and 9 are ER+/PR+.

For the quantification of ERC4 variant mRNA, and as underlined in our previous report, we have redesigned our triple-primer polymerase chain reaction (TP-PCR) assay that we had previously set up to quantify ERC4 mRNA expression.¹⁴ This new TP-PCR, that uses new primers, has been validated by comparing the results obtained with those obtained using a standardized RNase protection assay (see Figure 3, **APPENDIX 4**).²¹ We showed that this approach is reliable and highly specific, and can be used to address the question of the expression of ERC4 variant mRNA relative expression in ER negative samples or samples having a very low ER, by binding assay. The expression of ERC4 mRNA has therefore been investigated using this new TP-PCR assay in the selected subset of 18 patients. For each patient, total RNA was extracted from the normal and tumor components of 20 μ m frozen cryostat sections. Reverse transcription of total RNA using random hexamers was followed by PCR amplification using three primers and dCTP [α -³²P]. PCR products were separated on 6% polyacrylamide gels and following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with an intensifying screen.

For each analysis, quantification of signals was carried out after excision of the bands corresponding to ERC4 and WT-ER- α mRNA (using autoradiographs as a guide), followed by addition of 5 ml scintillant (ICN Pharmaceuticals, Inc, Irvine, California) and counting in a scintillation counter (Beckman). The ratio between ERC4 and WT-ER- α signals was calculated. For each experiment, the ratio observed in the same particular tumor (case number 12), was arbitrarily attributed the value of one and all other ratios expressed relatively. This normalization eliminates any possible variation resulting from different inter-experimental parameters, such as batches of radio-label, Taq polymerase etc... Results obtained are summarized in Figure 1 of **APPENDIX 3**.

Detection, analysis and quantitation of ERD3 and ERD5 mRNAs was performed similarly but using primers annealing with exons 2 and 4, and exons 4 and 6 of the WT-ER- α , respectively. Results obtained are presented in Figure 2 (ERD3) and Figure 3 (ERD5) of **APPENDIX 3**.

We concluded that a general trend towards a higher ERC4 mRNA and a lower ERD3 mRNA relative expression in the tumor compartment was observed. These differences reached statistical significance when considering only the ER+/PR+ ($p=0.019$) and the ER+ ($p=0.023$) subsets, as measured by the ligand binding

assay, respectively. A significantly ($p=0.035$) higher relative expression of ERD5 mRNA was observed in tumor components overall. These data, which confirm previous observations obtained on independent normal and tumor breast tissues, demonstrate that changes in the expression of ER- α variant mRNAs relative to WT-ER- α occur between adjacent normal and neoplastic breast tissues.

We then addressed the question of the expression of ER- α variant mRNAs during the transition from primary to metastatic breast cancer. Using similar RT-PCR assays, we examined the relative expression of ERC4, ERD5 and ERD7 variant mRNAs in 15 primary breast tumors and in their matched concurrent axillary lymph node metastases. Overall, there were no significant differences between the primary tumors and the matched metastases (see Figure 2 **APPENDIX 4**).²¹

Objective 2: To identify variant ER mRNAs differentially expressed in normal breast and breast cancer tissue.

Using targeted PCR, all ER variants previously identified in breast tumors were detected in normal breast tissue (ie ERD3, ERD4, ERD5, ERD7 and ERC4). This suggested that multiple ER variant mRNAs are expressed in both normal and tumor breast tissues. We have developed a strategy (called long-range PCR) to allow the investigation of full length known and unknown exon-deleted or inserted ER variant mRNAs in any one tissue sample. This approach allows the determination of any change which could occur in the relative expression of such variants amongst themselves and with respect to WT-ER- α transcript (**APPENDIX 5**).²² Briefly, cDNAs corresponding to all exon-deleted ER variants are amplified together with the WT-ER- α mRNA using primers annealing with exon 1 and exon 8 sequences. A competitive amplification occurs amongst all exon-deleted or inserted ER variant transcripts, that depends on their initial relative representation.

This approach has been used successfully to identify exon 3-4-deleted variant mRNA as differentially expressed between an estrogen sensitive cell line (T5) and an estrogen non-responsive cell line (T5-PRF), obtained by chronically depleting T5 cells of estrogen in long term culture (see Figure 3, **APPENDIX 6**).²³ One should note that this long-range PCR approach has also been successfully used to identify new PR receptor mRNA variants in breast tumor tissue (**APPENDIX 7**).²⁴ One of these variants, deleted in sequence encoding exon 6, was found to be more highly expressed in breast tumor tissues than in independent normal breast tissues (**APPENDIX 8**).²⁵

Long range PCR analyses have been performed on matched normal and tumor samples. This approach failed to give reproducible results when performed on normal breast tissue samples. The lack of (and in most of the cases absence of) reproducible signals, already observed for ER negative breast tumor samples (see Figure 2, **APPENDIX 5**),²² as assessed by ligand binding assay, is likely to result from the low level of expression of

ER- α in normal breast tissues. Indeed, it is likely that the low number of ER- α cDNA molecules does not allow the corresponding long PCR products to be reproducibly and efficiently amplified during the first cycles of the PCR. We tried, with no success, to overcome this difficulty by increasing the amount of input cDNA. Indeed, the low amount of starting material available (i.e frozen sections from normal breast tissue adjacent to breast tumor) rapidly limited the quantity of cDNA we could obtain. The fact that it was not possible to use long-range PCR to investigate the expression of full length ER- α variant mRNA population in normal breast tissue resulted in our inability to fully complete our second objective and subsequently slowed down the realization of the third one. Indeed, at that stage of our study, the only information we were able to gather with experimental confidence, regarding the expression of exon-deleted ER- α variant mRNA in matched normal and tumor tissues, was obtained using targeted PCR (performed using primers spanning only a small limited region of the recognized cDNAs). Therefore, as developed in the discussion of the **APPENDIX 9**,²⁶ while the relative proportion of the regions spanned by the set of primers can be determined, no data are provided on the other regions of the recognized cDNAs. In other words, even though we are confident that changes in the balance between ER- α related molecules is different between matched normal and tumor breast tissues, we cannot determine with certainty the identity of the variants involved. For example, although the relative expression of ERD5 is higher in tumor versus normal tissue, our assay measures all cDNAs deleted in exon 5 sequence, some of which may correspond to full length ERD5 mRNA but some of which may be deleted in other exons outside the amplified regions. This important limitation concerning the real identity of the full-length cDNA molecules detected using targeted PCR had to be considered before starting the third objective.

Objective 3: To determine the putative function of differentially expressed variants.

For the reasons mentioned above, this objective has not been completed.

Objective 4: Does the expression of particular ER- α variant mRNAs differ between the normal breast tissue of women with and without diagnosed breast cancer ?(APPENDIX 9)²⁶

In order to fulfill this objective, we started a collaboration with Dr. Seema Khan, surgeon in the Department of Surgery of the University Hospital, Syracuse, NY. Nineteen case and eighteen control subjects in the luteal phase of their menstrual cycle at the time of surgery were selected from a previously analyzed cohort.²⁷ Case subjects were women with newly or previously diagnosed *in situ* (n = 6) or invasive (n= 13) breast cancer who required further surgery. Six case samples were obtained from mastectomies whereas thirteen samples came from biopsies. Control subjects were women without a prior history of breast cancer who required diagnostic breast biopsy, which proved not to be breast cancer. The lesions observed in control women were fibroadenoma (n = 8), fibrocystic diseases (n = 3), hyperplasia (n = 2), fibrosis (n = 3) and intraductal papilloma (n = 1). The remaining control sample was obtained from reduction mammoplasty. The phase of the

cycle was established by questioning patients at the time of surgery. The presence of normal ducts and lobules as well as the absence of any atypical lesion in all normal tissue specimens was confirmed by histopathological examination of H&E stained tissue sections. Total RNA was extracted from frozen breast tissue sections using Trizol™ reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions, and quantified spectro-photometrically. One µg of total RNA was reverse-transcribed in a final volume of 25 µl as previously described.¹⁴ The quality of the cDNAs obtained was checked by PCR amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The expression of ERD5, ERD7 and ERC4 mRNA have been measured by the means of targeted RT-PCR performed using primers depicted Figure 1, **APPENDIX 9**. Figure 2 of **APPENDIX 9** shows that a significantly higher (Mann-Whitney rank sum test, $p < 0.02$) expression of ERD5 variant mRNA relative to WT-ER mRNA was observed in the normal breast tissue of controls (median 20.35%, $n = 16$) as compared to cases (median = 10.97%, $n = 19$). The levels of expression of ERD7 and ERC4 variant mRNAs were found to be similar in the normal breast tissue of both subgroups of patients (Figures 3-4, **APPENDIX 9**). As discussed above, whether or not the change observed in ERD5 mRNA expression reflects a modification of the expression of the full length ERD5 mRNA is unknown. However the data support a modification of the balance existing between the different ER- α like mRNAs present in the normal breast tissue of cases and controls. This suggests that ER- α signaling pathway may differ in the normal breast tissue of the two groups. As mentioned earlier in the text of this report, the morphologically normal breast tissue adjacent to breast tumor has been shown to contain some genetic abnormalities. Indeed, Deng et al. reported a loss of heterozygosity in 3p22-25 in morphologically normal lobules adjacent to breast cancers.¹⁵ Because some of the case samples in this study were obtained from biopsies and therefore came from normal tissue adjacent to the tumor, one cannot exclude that the difference in the expression of ERD5 mRNA between cases and controls results from the vicinity of tumor tissue. Whether or not the differences observed pre-exist or result from the presence of breast cancer remains to be determined.

Objective 5: Is ER- β expressed in human breast tumors ? (APPENDIX 10)²⁸

The expression of ER- β mRNA has first been investigated in human breast biopsy samples and several human breast epithelial using RT-PCR performed with primers annealing with exon 7 and exon 8 of WT-ER- β 1 (**APPENDIX 10**, also see **APPENDIX 2** for the definition of ER- β 1). As shown in Figures 1 and 2 (**APPENDIX 10**), ER β 1 is detected in some but not all samples analyzed. Indeed, ER- β expression was not correlated with that of ER- α , and both known ER+ and ER- cell lines expressed ER- β mRNA. However, some cell lines and some tumors co-expressed both receptors. Because the level of expression of

ER- β mRNA varied widely from one tumor to another, it was interesting to investigate the possible relationship between ER- β expression and steroid receptor status in breast tumors.

Objective 6: What is the relationship between ER- β expression and steroid receptor status in breast tumors ?(APPENDIX 11)²⁹

In order to fulfill this objective, forty invasive ductal carcinomas were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected for ER and PR status as determined by ligand binding assays. Ten tumors were classified as ER+/PR+ (ER range 50 - 127 fmol/mg protein, PR range 105 - 285 fmol/mg protein); 10 tumors were classified as ER+/PR- (ER range 59 - 156 fmol/mg protein, PR range 5 - 10 fmol/mg protein); 10 tumors were ER-/PR- (ER range 0 - 2 fmol/mg protein, PR range 0 - 10 fmol/mg protein); 10 tumors were classified as ER-/PR+ (ER range 5 - 9 fmol/mg protein, PR range 51 - 271 fmol/mg protein). These tumors spanned a wide range of grade (grade 4 to 9), determined using the Nottingham grading system. Total RNA was extracted and reverse-transcribed as previously described.¹⁴ ER- β cDNA was amplified in the presence of dCTP [α -³²P] using primers annealing with exon 1 and 2 of ER- β sequence (see Figure 1A, **APPENDIX 11**). Results obtained are shown Figure 1 and 2 of **APPENDIX 11**. When the level of ER- β mRNA in tumors was assessed according to either ER status or PR status alone, the level of ER- β mRNA was significantly lower in PR+ tumors compared to PR - tumors ($p=0.036$) and no association with ER status was found. Subgroup analysis showed that ER- β mRNA expression in ER+/PR+ breast tumors was significantly less than in ER+/PR- ($p=0.009$), ER-/PR+ ($p=0.029$) and ER-/PR- ($p=0.023$) groups (see Figure 1C, **APPENDIX 11**). Our data suggested that the expression of ER- β in breast tumors could be a marker of endocrine therapy responsiveness. Interestingly, the ER- β mRNA expression was specifically decreased by progestin in T-47D breast cancer cells (see Figures 2 and 3, **APPENDIX 11**).

Objective 7: Do equivalent ER- β variants exist ? (APPENDIX 12, 13).^{30, 31}

Long range PCR performed using ER- β primers annealing with sequences in exon 1 and exon 8 of ER- β gene allowed us to identify an exon 5-6-ER- β variant in human breast tumors (**APPENDIX 12**).³⁰ This observation was the first observation of exon-deleted ER- β variants and we suggested that, as with ER- α variants, ER- β variants might also have a role in the mechanisms underlying hormonal progression in breast cancer. Since then, we have identified using targeted RT-PCR analysis of RNA extracted from normal as well as tumor breast tissue several other ER- β mRNA isoforms deleted in exon 5 or exon 6 (**APPENDIX 13**).³¹ Interestingly, one should note that inter-species differences exist in the expression of ER- β (see Figure 3, **APPENDIX 13**) and ER- α (see Figure 2, **APPENDIX 14**)³² variant mRNAs. These interspecies differences suggest that, depending on the functional significance of ER- β and ER- α variants (that indeed

remains to be determined), the extrapolation of results of some estrogen related studies in the mouse model to the human might be problematic.

The existence of similar as well as several other variants deleted in exon 8 sequences and putatively able to encode C-terminally truncated proteins has now been documented by other groups (for a review, see **APPENDIX 2**).

Objective 8: Is the relative expression of some of these variants modified during breast cancer progression and tumorigenesis ? (APPENDIX 15)³³

We have developed a triple primer polymérase chain reaction (TP-PCR) assay to evaluate the relative expression of ER- β 1, ER- β 2, ER- β 4 and ER- β 5 variant mRNAs. As shown in Figure 1A (**APPENDIX 15**), ER- β 1, ER- β 2, ER- β 4 and ER- β 5 mRNAs all have exon 7 sequences, but differ from each other in the following sequences. Interestingly, comparison of the sequences revealed that ER- β 2, ER- β 4 and ER- β 5 mRNAs have sequence similarities within their 3' extremities. Therefore, it was possible to use the previously described TP-PCR to investigate the relative expression of these variants. Three primers were designed (ER- β 1U, ER- β 1L and ER- β 2L) which recognized exon 7 sequences common to all transcripts, ER- β 1 exon 8 specific sequences, and sequences shared by ER- β 2, ER- β 4 and ER- β 5 mRNAs, respectively (see Figure 1A, **APPENDIX 15**).

The assay was tested using several different human breast cancer cell lines (Figure 1B, **APPENDIX 15**) and validated on spiked DNA preparations containing different relative amounts of ER- β 1, ER- β 2, and ER- β 5 PCR products (Figure 2, **APPENDIX 15**).

This assay was used to evaluate the relative expression of ER- β 1, ER- β 2 and ER- β 5 mRNA within 53 breast tumors (characteristics of which are indicated in the "Materials and Methods" section of **APPENDIX 15**) and in some cases (n=13), within adjacent normal breast tissue. The expression of ER- β 2 and ER- β 5 mRNAs was higher than that of ER- β 1 mRNA in both cancer cell lines and breast tumors (see Figures 2 and 3, **APPENDIX 15**). In breast tumors, an increase in the ratio of ER- β 2/ER- β 1 and ER- β 5/ER- β 1 mRNA expression was observed, that positively correlated with the level of tumor inflammation and tumor grade, respectively (Figure 3, **APPENDIX 15**). A trend towards an increase of these ratios was also found in tumors, as compared to the normal adjacent breast tissue available for 13 cases (see Figure 4, **APPENDIX 15**). These data suggest that changes in the relative expression of ER- β 1, ER- β 2, and ER- β 5 mRNAs occur during breast tumorigenesis as well as during tumor progression.

It is now clear that the ER- β signaling pathway, as with that of the ER- α , is complex and will likely include the interaction between several ER- β isoforms. Therefore, the balance between the ER- α and ER- β related

molecules may be an important parameter to consider when studying the action of estrogens on both normal and neoplastic mammary tissue.

Objective 9: Does the balance between ER- α and ER- β change during breast tumorigenesis ? (APPENDIX 16)³⁴

In order to establish whether changes occur in the balance of ER- α and ER- β receptor during breast tumorigenesis, we designed a multiplex RT-PCR assay that allows the relative ER- α /ER- β content to be measured in small tissue samples. The regions co-amplified in the PCR reaction consist of a region encompassing exon 1 and exon 2 sequences of ER- β , and exon 2 and exon 3 for ER- α . This assay has been validated using spiked cDNA preparations (Figures 1 and 2, **APPENDIX 16**) and has been used to study the relative ER- α /ER- β expression in the subset of 18 normal breast tissues and their matched 18 breast tumor samples mentioned above. No differences in the ratio ER- α /ER- β were observed in the ER- cohort. However, a significantly ($p < 0.02$) higher ER- α /ER- β ratio was observed in the ER+ tumors compared with that of their matched adjacent normal component (see Figure 4, **APPENDIX 16**). This increase was attributed to a significantly ($p < 0.05$) increased ER- α mRNA expression, often in conjunction with a lower ER- β mRNA expression in the tumor compared with that of the normal component. Our results suggest that the role of ER- α and ER- β driven pathway and/or their interaction change during human breast tumorigenesis.

Objective 10: Is SRA expressed in breast tumor tissues ? If yes, is this expression changed during breast tumor progression? (APPENDIX 17)³⁵

The expression of the recently described steroid receptor RNA activator (SRA) was measured by semi-quantitative reverse-transcription polymerase chain reaction within 27 independent breast tumors, spanning a wide spectrum of grade, estrogen receptor (ER) and progesterone receptor (PR) levels (see Materials and Methods section, **APPENDIX 17** for tumor characteristics). In order to investigate the expression of all described SRA isoforms in human breast tumor tissues, we designed primers to amplify a 662 bp long fragment encompassing almost all the SRA core region (the primers used are depicted Figure 1, **APPENDIX 17**).

Total RNA was extracted, reverse-transcribed, and PCR amplification was performed as described in the "Materials and Methods" section (**APPENDIX 17**), using SRA core primers. A 662 bp long fragment was obtained in all samples (see Figure 2, **APPENDIX 17**). This fragment was sequenced and corresponded to the SRA core region. An additional fragment, migrating at an apparent size of 459 bp was also observed in most samples. Sequencing analysis revealed that this band corresponded to a variant form of SRA (referred to as SRA-Del) deleted in 203 bp between positions 155 and 357 (positions given correspond to Genbank accession number AF092038).

For each case, SRA corresponding signal was quantified and expressed in arbitrary units. Results obtained from the 27 cases, grouped according to their ER and PR levels, as determined by ligand binding analysis, are presented Figure. 3A, **APPENDIX 17**. When the cohort of cases was considered as a whole ($n = 27$), no correlation was observed between SRA expression and ER or PR levels. However, when only ER- tumors were considered ($n = 13$), a trend toward a positive correlation between SRA expression and PR levels was observed (Spearman coefficient $r = 0.527$, $p = 0.064$). Indeed, a statistically significant (Mann-Whitney rank sum test, two sided, $p = 0.045$) higher SRA expression was observed in ER-/PR+ ($n = 8$, median = 144.8) than in ER-/PR- tumors. In contrast, within ER+ cases ($n = 14$), SRA expression negatively correlated with PR levels (Spearman coefficient $r = -0.810$, $p = 0.0004$). SRA expression was indeed higher (Mann-Whitney rank sum test, two sided, $p = 0.001$) in ER+/PR- ($n = 6$, median = 156.4) than in ER+/PR+ cases. In a similar way, SRA expression correlated positively (Spearman coefficient $r = 0.735$, $p = 0.009$) and negatively (Spearman coefficient $r = -0.532$, $p = 0.033$) with ER levels in PR- ($n = 11$) and PR + ($n = 16$), respectively. SRA levels were higher in ER+/PR- than in ER-/PR- tumors (Mann-Whitney rank sum test, two sided, $p = 0.017$) and in ER-/PR+ than in ER+/PR+ cases (Mann-Whitney rank sum test, two sided, $p = 0.047$). SRA levels of expression did not correlate with tumor grade scores (Figure. 3B, **APPENDIX 17**).

SRA-Del levels strongly correlated (Spearman coefficient $r = 0.530$, $p = 0.004$) with Nottingham grade scores within the whole cohort ($n = 27$). Indeed, the level of expression of SRA was significantly higher (Mann-Whitney rank sum test, two sided, $p < 0.05$) in tumors with high grade ($n = 7$, median = 6.572) than in tumors with low ($n = 4$, median = 2.192) or intermediate ($n = 9$, median = 2.588) grade (Figure. 4B, **APPENDIX 17**).

In conclusion we showed that SRA is expressed in breast tumors and that its expression correlates with ER and PR levels in particular tumor subgroups. We speculate that changes in SRA expression could be involved in the mechanisms underlying tumor progression and hormone resistance.

Objective 11: Is SRA expressed in normal breast tissue ? If yes, do changes occur in SRA level of expression during breast tumorigenesis ? (APPENDIX 18)³⁶

Using the same reverse transcription polymerase chain reaction assay, the expression of SRA was compared between adjacent normal human breast tissue and matched breast tumors from 19 patients (characteristics of the tumors are detailed in the Materials and Methods section, **APPENDIX 18**). Core SRA RNA was detected in normal and neoplastic breast tissues (see Figure 2, **APPENDIX 18**). The level of SRA RNA was significantly ($p = 0.0004$) higher in breast tumors than in matched normal breast (see Figure 3, **APPENDIX 18**). SRA-Del RNA was also detected in most samples of normal breast and tumors (see Figure 3, **APPENDIX 18**). No differences occurred in the relative expression of the deleted SRA between normal breast and tumors. Our data suggested that expression of core SRA is up-regulated during breast

tumorigenesis and that changes in the relative expression of a deleted SRA isoform occur during breast cancer progression.

KEY RESEARCH ACCOMPLISHMENTS

Objectives 1, 2: A higher expression of ERD5 and ERC4 variant mRNAs in the breast tumor component compared to the normal counterpart of matched samples was observed. Inversely, a higher expression of ERD3 variant in the normal component compared to the tumor component of the same matched cases was found. This is consistent with previous observations made on independent samples. There were no significant differences in the expression of ERC4, ERD5 and ERD7 variant mRNAs between primary tumors and matched metastases.

Objective 4: A significantly higher expression of ERD5 variant mRNA relative to WT-ER mRNA was observed in the normal breast tissue of controls (women without breast diagnosed cancer) as compared to cases (women with breast cancer). The levels of expression of ERD7 and ERC4 variant mRNAs were found to be similar in the normal breast tissue of both subgroups of patients.

Objective 5, 6: ER- β mRNA is expressed in breast tumors, together with an exon-3-deleted variant mRNA. The expression of ER- β mRNA in ER+/PR+ breast tumors was significantly less than in ER+/PR- , ER-/PR+ and ER-/PR- groups. Interestingly, the ER- β mRNA expression was specifically decreased by progesterin in T-47D breast cancer cells.

Objective 7: We have described the presence within normal breast as well as in breast tumor tissues of several variant forms of ER- β mRNA deleted in exon 5, exon 6 and in exon 5+6 or exon 8 sequences. Inter-species differences exist in the expression of ER- β and ER- α variant mRNAs.

Objective 8: The expression of ER- β 1, ER- β 2, and ER- β 5 mRNAs is modified during breast tumorigenesis and tumor progression.

Objective 9: We showed that a significantly higher ER- α /ER- β ratio was observed in the breast tumors compared with their matched normal breast tissues. This increase was attributed to a significant increase in ER- α mRNA expression and a lower ER- β mRNA expression in the tumor compared with that of the normal component in some ER+ cases.

Objective 10, 11: SRA is expressed in normal and tumor breast tumor tissues together with a variant form deleted in exon 3 sequence (SRA-Del). SRA expression correlated with ER or PR levels in particular tumor subgroups. The level of SRA RNA was significantly higher in breast tumors than in matched normal breast tissue. SRA-Del expression strongly correlated to the grade of the tumors.

REPORTABLE OUTCOMES

List of articles and communications reporting the results of these studies. All these reports have been included as appendices.

Articles and reviews published or in press in peer reviewed journals

- 19 Coutts, A. S., Leygue, E., and Murphy, L. C., Variant estrogen receptor alpha messenger RNA in hormone independent human breast cancer cells. *J Mol Endocrinol*, In press. **APPENDIX 6**
- 18 Lu, B., Dotzlaw, H., Leygue, E., Murphy, L.J., Watson, P. H., and Murphy, L. C. Estrogen receptor alpha mRNA variants in murine and human tissues. *Mol Cell Endocrinol*, In press. **APPENDIX 14**
- 17 Leygue, E., Dotzlaw, H., Watson, P. H., and Murphy, L. C. Expression of the steroid receptor RNA activator (SRA) in human breast tumors. *Cancer Res*, In press. **APPENDIX 17**
- 16 Huang, A., Leygue, E., Dotzlaw, H., Murphy, L. C., and Watson, P. H. Estrogen receptor mRNA variants influence the determination of ER status in human breast breast cancer. *Breast Cancer Res Treat*, In press. **APPENDIX 20**
- 15 Leygue, E., Dotzlaw, H., Lu, B., Watson, P. H. and Murphy, L. C. Estrogen receptor beta: a review. *Cur Top Steroid Res*, In press. **APPENDIX 2**
- 14 Leygue, E., Dotzlaw, H., Watson, P. H., and Murphy, L. C. Altered expression of exon 6 deleted progesterone receptor variant mRNA between normal human breast and breast tumor tissues. *Brit J Cancer*, 80: 379-382, 1999. **APPENDIX 8**
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Articles submitted to peer reviewed journals

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- 1 Leygue, E., Dotzlaw, H., Watson, P. H., and Murphy, L. C. Altered expression of estrogen receptor alpha variant mRNAs between adjacent normal breast and breast tumor tissues. Submitted, Breast Cancer Res. **APPENDIX 3**

Book chapters

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Abstracts and oral communications

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- 11 Dotzlaw, H., Leygue, E., Watson, P. H., and Murphy, L. C. Expression of estrogen receptor- α (ER- α) and estrogen receptor-beta (ER- β) mRNA in primary human breast tumors and matched normal human breast tissue samples. 10th International Congress of Hormonal Steroids. Quebec, Quebec, Abstract 63, 1998. **APPENDIX 31**
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CONCLUSION

In conclusion, we showed that in normal breast tissue, the expression of ER- α and ER- β variant mRNAs, deleted in regions encoding functional domains of WT-ER- α and WT-ER- β , was different than that observed in adjacent breast tumor tissue. In the future, it will be important to determine whether or not these changes in mRNA expression are transposed at the protein level. Even though a recent concern has been expressed with respect to the significance of ER-like mRNAs, several groups reported data to support the translation of the predicted ER-like proteins *in vivo* (for a review, see **APPENDIX 2** and **APPENDIX 19**).³⁷ Recently, a group of breast tumors were analyzed immunocytochemically for ER- α expression using antibodies which recognized either an N-terminal or a C-terminal epitope of the WT-ER- α protein.³⁸ Even though in many tumors (consistent tumors) similar signals were obtained using both antibodies, It was found that in some tumors (inconsistent tumors), the results were discordant, with a signal tending to be higher when using the N-terminal antibody. Since many of the proteins predicted from variant ER- α mRNAs would be missing the C-terminus extremity of WT-ER- α protein, one interpretation would be that truncated ER- α proteins are more highly expressed in the inconsistent than in the consistent tumors. We have indeed shown that cells transfected with ERC4 expression vector presented discordant results when analyzed immunocytochemically using antibodies recognizing N- and C-epitope, as opposed to cells transfected with WT-ER- α expression vector (**APPENDIX 20**).³⁹ We have investigated the pattern and relative expression of variant ER- α mRNAs in the consistent and inconsistent groups of breast tumors (**APPENDIX 21**).⁴⁰ We showed that, whereas overall prevalence of ER- α variant mRNAs was similar in both tumor groups, occurrence of the subset of variant mRNAs encoding putative truncated proteins was higher in inconsistent tumors than in consistent ones. While this indirect approach did not specifically identify ER- α variant proteins, the data suggested that the ER- α variant mRNAs encoding truncated ER- α proteins may contribute to discrepancies in ER- α measured by immunodetection assays using N- or C-terminal antibodies. Only the use of antibodies recognizing specifically the unique C-terminal extremity of the encoded variant proteins will allow the

determination of whether the changes in the expression of ER- α and ER- β variant mRNAs observed between normal and tumor tissues are indeed transposed at the protein level.

So what ?

It is reasonable to assume that ER signaling pathway involves a complex population of ER- α and ER- β like molecules and that the balance between their respective expression modulates the action of estrogens as well as antiestrogens. In a time when tamoxifen, already used in hormone therapy, is going to be used to prevent breast cancer in women at risk,⁴¹ it is crucial to understand the mechanisms underlying antiestrogen action in normal and tumor breast tissues. Only the knowledge of all the players involved together with the changes in their expression occurring during tumorigenesis and breast tumor progression will provide the bases to understand such mechanisms and their relevance *in vivo*. The change in the expression of SRA observed during breast tumorigenesis and tumor progression suggests that co-activator molecules, that will also modulate the action of estrogen and antiestrogens, should be considered in the future studies aiming to increase our knowledge of the mechanisms involved in these processes.

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APPENDICES

APPENDIX 1

Murphy LC, **Leygue E**, Dotzlaw H, Douglas D, Coutts A, and Watson PH

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Oestrogen Receptor Variants and Mutations in Human Breast Cancer

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Several oestrogen receptor variant and mutated mRNA species have been identified in human breast samples and cell lines. Over-expression and altered expression of some of these mRNAs have been correlated with breast tumourigenesis and progression. The following review focuses on the current knowledge available in the scientific literature with respect to the type and characteristics of oestrogen receptor variants and mutations that have been identified as occurring naturally in human breast tissues and cell lines.

Key words: breast cancer; mutations; oestrogen receptor.

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Introduction

Oestrogens are major regulators of mammary gland development. However, oestrogens are also involved in the growth and progression of mammary cancers (1). The principal mechanism by which the effects of oestrogen are mediated in either normal or neoplastic target cells is via an initial interaction with the oestrogen receptor (ER). This protein is an intracellular ligand-activated transcription factor regulating the expression of several gene products, which ultimately result in target-tissue-specific oestrogen responses. The ER can be divided into several domains, labelled A–F, starting from the N-terminus (2, 3). Mutational analyses have defined several functional regions within each domain. The A/B region contains a cell- and promoter-specific, ligand-independent nonacidic transactivating function (AF-1), which may have a role in the agonist activity of the tamoxifen-like antioestrogens (4–6). The C domain contains two zinc finger motifs, which are responsible for the specific DNA-binding activity of the protein (2, 3). The C domain also contains an apparently constitutive dimerization domain (2). The D domain is thought to be

a flexible hinge region but also contains a number of basic amino acids conserved in all receptors, which may have a role in nuclear localization and DNA binding (2). The E domain contains the ligand-binding domain, a ligand-dependent dimerization activity and a ligand-dependent nonacidic transactivating function (AF-2). The carboxy terminal F domain was originally thought to have no functional significance; however, more recent analyses suggest that it has a specific modulatory function on transcriptional responses to oestrogens and antioestrogens that is influenced by cell context (7). Upon oestrogen binding the receptor undergoes conformational changes resulting in its 'activation', so that it forms stable homodimers that bind tightly to specific nucleotide sequences called oestrogen-responsive elements, or EREs (2, 3). EREs are usually found in the promoter region of those genes the transcription of which is regulated by oestrogen. In this way oestrogen can alter the transcription of several genes that ultimately lead to DNA synthesis and proliferation of breast cancer cells.

However, the involvement of oestrogen in mammary tumour growth and progression is thought to involve, at some stages, perturbations of the ER signal transduction pathway, which are likely to contribute to tumour progression and the eventual development of hormone independence and a more aggressive phenotype (8–10). One mechanism underlying such perturbations could be alterations in the structure and therefore function of the ER itself. This review will focus on structural changes in the ER that have been identified as occurring naturally in human breast tissues and cell lines.

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Identification and Structure of ER Variant and Mutant mRNA Species

Molecular evidence for the potential existence of variant and/or mutant ER proteins has been obtained by analysis of ER-like mRNA in normal and neoplastic breast tissues. Many different types of ER-like mRNAs distinct from the wild-type ER mRNA have now been identified in several tissues and cell lines, including both normal and neoplastic human breast cells and tissues. It should be remembered, however, that few of these ER-like transcripts have been cloned and characterized from cDNAs representing full-length transcripts. Indeed little, if any, attention has been paid to the presence or absence of a 3'-untranslated region, a polyadenylation signal and a poly A tail. Given these caveats to interpretation, several different patterns of ER-like mRNA have been found or predicted, as described in the following.

Transcripts Containing Precise Single or Multiple Exon Deletions

Multiple ER-like transcripts have been identified that contain precise exon deletions (11–20). Several of the exon-deleted transcripts that have been described in the literature are shown in Figure 1. The majority of these have been identified by reverse transcription-polymerase chain reaction (RT-PCR) approaches, which by virtue of specific primer design have focused on small regions of the known wild-type ER mRNA. More recently, however, ER-like transcripts containing two or three entire exon deletions have been detected in cell lines and tissue samples (16–20) and amongst the deletion-type ER variants, this type of variant ER transcript appears now to be the most predominant. However, the identification of multiple types of exon deleted transcripts in any one cell line or tissue sample (16–18, 20) underscores the need to study these variant ER transcripts altogether, as well as individually.

Other Deleted Transcripts

ER-like transcripts containing variable-sized deletions that are not entire exon deletions have also been detected. This type of alteration falls into two groups: one in which a single nucleotide has been deleted (21, 22), and the other in which several hundreds of continuous nucleotides have been deleted but starting and ending within known exon sequences (Fig. 1, Table 1) (16, 20, 21, 23).

Truncated Transcripts

These altered ER-like transcripts are significantly smaller than the wild-type ER mRNA as determined by Northern blot analysis (24). cDNA cloning of apparently full-length or near to full-length transcripts was used to characterize these transcripts fully. These transcripts contain entire exon sequences of at least 2 of the 5' ER exon sequences, and then diverge into ER-unrelated sequences (25), some of which appear to be LINE-1

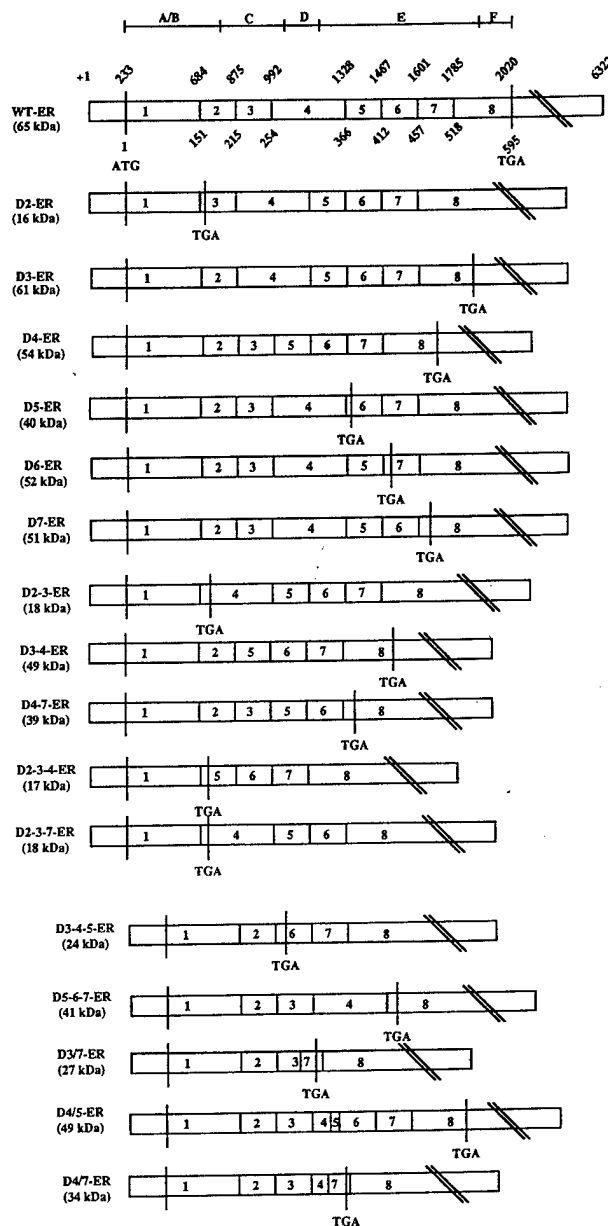


Figure 1. Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F). Region A/B of the receptor is implicated in transactivating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (AF-2). The numbering on the top of the cDNA refers to the nucleotide position as defined in (64). Below the WT-ER cDNA are the various putative exon and other large deleted ER cDNAs. ATG shows the translation initiation codons, TGA shows the inframe translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in (64). D indicates deletion, and the estimated molecular mass (kDa=kiloDaltons) of each open reading frame is shown in parentheses. Molecular masses were estimated using MacVector version 4.1.4 software.

Table 1. ER variants identified in human breast tissues and cell lines.

Variant mRNA	Estimated M_r of predicted protein (kDa)	Functional domains	Reference
Wild-type ER	65	A, B, C, D, E, F	(4, 5, 64)
D2-ER	16	A, B?	(11, 16–19)
D3-ER	61	A, B, D, E, F	(11, 14)
D4-ER	54	A, B, E?, F	(12, 17–20, 59)
D5-ER	40	A, B, C, D	(13)
D6-ER	52	A, B, C, D	(14)
D7-ER	51	A, B, C, D	(11, 15, 20)
D2-3-ER	18	A, B?	(16, 20)
D3-4-ER	49	A, B, E?, F	(16, 17, 20)
D4-7-ER	39	A, B	(18–20)
D2-3-4-ER	17	A, B?	(20)
D2-3-7-ER	18	A, B?	(20)
D3-4-5-ER	24	A, B	(18)
D5-6-7-ER	41	A, B, C	(18)
D3/7-ER	27	A, B	(20)
D4/5-ER	49	A, B, C?, F	(21)
D4/7-ER	34	A, B, C?	(23)
Clone 4-ER	24	A, B	(25)
Clone 24-ER	37	A, B	(25)
Exon 6 ² -ER	51	A, B, C, D	(26)
Exon (34) ² -ER	75	A, B, C+, D+, E, F	(26)
Exon (67) ² -ER	80	A, B, C, D, E+, F	(27, 45)
ER-69-bp	69	A, B, C, D, E?, F	(26, 28)

? Indicates that an alteration of the function has been shown or is likely to occur.

related (Fig. 2, Table 1). Although several different truncated ER mRNAs have been cloned, some of these were only found to be expressed in a single breast tumour, although others, such as the clone 4-truncated ER mRNA, have been found to be expressed in many human breast tumours (25).

Insertions

ER-like transcripts have been identified containing variable-sized nucleotide insertions. Such insertions consist of one to two nucleotides (21, 22), larger insertions of 69 and more nucleotides (21, 26), and apparently complete exon-duplications (26, 27) (Fig. 2, Table 1). These abnormal ER-like transcripts were detected using RT-PCR analyses, and further studies showed that the exon 6 plus 7-duplicated ER-like transcript was generated from a mutated ER gene in which genomic rearrangement resulted in the duplication of exons 6 and 7 in an in-frame fashion (27). As well the 69-bp-inserted ER mRNA is probably generated from a point mutation in one allele of the ER gene in the breast tumour from which it was cloned. This point mutation generates a consensus splice donor site at the 3' end of the 69-bp sequence present in intron 5. In addition, a splice acceptor consensus sequence is normally present at the 5' end of the 69-bp sequence, and thus the 69 sequences are likely to be seen as another exon in the gene (28).

Point Mutations

Several point mutations including silent polymorphisms have been identified in ER-like transcripts (Table 2) (21,

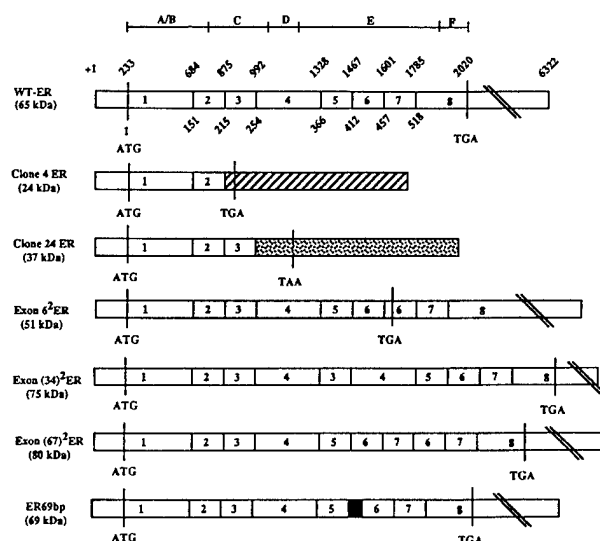


Figure 2. Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F), as described in Figure 1. The numbering on the top of the cDNA refers to the nucleotide position as defined in (64). Below the WT-ER cDNA are the clone 4- and clone 24-truncated ER cDNAs, which have been cloned previously (25), as well as the putative cDNAs representing exon-duplicated and some inserted ER mRNAs (26, 27). ATG shows the translation initiation codons, TGA shows the in-frame translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in (64). The estimated molecular mass (kDa=kiloDaltons) of each open reading frame is shown in brackets. Molecular masses were estimated using MacVector version 4.1.4 software.

Table 2. Small insertions/deletions and point mutations/polymorphisms identified in the oestrogen receptor mRNA.

Nucleotide change	Exon	Amino acid change	Functional domains	Reference
262 T→C	1	10 Ser no change	A, B, C, D, E, F	(25, 29)
439 C→G	1	69 Asn→Lys	A, B?, C, D, E, F	(29)
493 G→C	1	87 Ala no change (B variant)	A, B, C, D, E, F	(29–32)
701 C→T	2	+Stop after 156	A, B?	(36)
961 C→T	3	243 Arg no change	A, B, C, D, E, F	(29)
TT insert after 981	3	Met 250→Ile+stop	A, B	(21)
1059 C→T	4	276 Gly no change	A, B, C, D, E, F	(22)
1119 T→C	4	296 Leu→Pro	A, B, C, D?, E?, F	(32)
1207 C→G	4	325 Pro no change	A, B, C, D, E, F	(29, 32)
1283 G→T	4	352 Asp→Tyr	A, B, C, D, E?, F	(34, 35)
1290 A→T	4	353 Glu→Val	A, B, C, D?, E?, F	(22)
1418 A→G	5	396 Met→Val	A, B, C, D, E?, F	(29)
1463 G del	5	411 Asp→Thr+6 extra novel a.a.	A, B, C, D	(21)
del T at 1526	5	432 Ser→His+4 extra novel a.a.	A, B, C, D	(22)
1503–1550 replaced by 1380–1422	6	424 Ile→Arg+28 extra novel a.a.	A, B, C, D	(22)
1647 G→A	7	472 Lys no change	A, B, C, D, E, F	(22)
1747 C→G	7	505 Ala no change	A, B, C, D, E, F	(22)
1963 T→C	8	577 His no change	A, B, C, D, E, F	(22)
2014 A→G	8	594 Thr no change	A, B, C, D, E, F	(29)

Nucleotides are numbered according to the start site of transcription (+1) in (64). ? Indicates that an alteration of the function has been shown or is likely to occur.

22, 29–35). The only known germline mutation in the human ER associated with disease is a point mutation (36) identified in a young adult male presenting with osteoporosis, unfused epiphyses, continued linear growth in adulthood, and oestrogen resistance. Furthermore, only approximately 1% of primary breast tumours have point mutations in the ER gene (22, 29), which in some cases might be linked to hereditary breast cancer (37).

The above ER-like mRNA molecules have, in most cases, been identified in human breast cancer tissues or human breast cancer cell lines. However, data are now emerging showing that several of the exon-deleted and truncated transcripts are also expressed in multiple samples of normal human breast tissue (16–18). This suggests that the mechanisms for generating these transcripts are present in normal human mammary cells and therefore these transcripts are normal variants, and probably generated by an alternative splicing mechanism (38). It is less likely that the inserted transcripts and many of the amino acid altering point mutations are normal variants. There is a greater likelihood that such transcripts were generated from a mutated ER allele present in some human breast tumours (27, 28). In summary, a large body of molecular data exists to support at least the potential for the existence of variant or abnormal ER-like proteins in human breast cancer.

Expression of Multiple ER Variant mRNAs in Human Breast Tissues

The identification of several ER variant mRNAs in normal human breast tissues implies that either the variant

mRNAs or their respective proteins may have a normal role in ER signal transduction. Consequently, changes in the balance of ER-like molecules could perturb the ER signalling pathway and contribute to tumour progression. It has therefore become important to determine whether levels and the pattern of ER variant expression are different between normal and neoplastic breast tissues, as well as amongst groups of tumours with different characteristics.

This has been studied initially by investigating individual variant ER mRNA levels relative to wild-type ER mRNA levels. The relative expression of the truncated clone 4 ER mRNA (39) and the exon 5-deleted ER mRNA, but not the exon 7-deleted ER mRNA (16) were found to be significantly elevated in breast tumour tissue compared with normal breast tissue. It has also been suggested that the level of the exon 3-deleted ER mRNA is reduced in breast tumour tissue compared with normal tissue (40). Such data suggest that the expression of some but not all variant ER mRNAs is deregulated during breast tumorigenesis.

Investigation of the relative expression of the truncated clone 4 ER variant in groups of breast tumours with different prognostic characteristics (41) identified a statistically significant increased expression of this transcript in breast tumours with combined characteristics of poor prognosis (node positive, large tumour size, high S-phase fraction) and lack of endocrine sensitivity (progesterone receptor (PR) negative). Elevated exon 5-deleted ER transcripts have been found in ER–/PR+ and ER–/pS2+ tumours (42), while increased levels of the exon 7-deleted ER mRNA are often found in ER+/PR– breast tumours (15).

These data suggest that altered expression of some ER variants is associated with different phenotypes in

human breast tumours and may have a functional role in such phenotypes. However, it has become increasingly apparent that several ER variant mRNAs can be detected in any one sample of either normal or cancerous breast tissues (16–18). While it is unclear whether any or all of these mRNAs are stably translated *in vivo* (see discussion below), many of the predicted ER-like proteins are lacking some functional domains (4) of the wild-type ER (Figs 1 and 2), and some have been shown to exhibit altered functions *ex vivo*. Therefore, the possibility exists that several ER variant proteins could be expressed together (16–18) and the validity of investigating individual variants in isolation can be questioned. Furthermore, previous analyses have depended largely on assays that focus on limited regions of the transcript, and would be unlikely to detect more than one modification per individual variant mRNA. However, it is now clear that more than one modification can occur in variant transcripts (19). Thus signals attributed to the exon 7-deleted ER variant mRNA, detected by RT-PCR using primers in exon 5 and 8 or by RNase protection assays with probes covering the exon 6/8 junction, may also include contributions from a variant deleted in both exon 4 and 7, recently identified by Madsen et al. (19). Nevertheless, these molecules may result in quite different proteins that differ in activity and may modulate differentially the ER signalling pathway. There is thus a need to investigate qualitatively and quantitatively the expression of total ER variant mRNAs within a single tumour. An attempt to address this issue was published recently (20). A strategy was developed to allow the investigation of known and unknown exon-deleted or inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants amongst themselves and with respect to the wild-type ER transcript. The approach (20) used is illustrated in Figure 3; however, owing to practical limitations it cannot measure all types of ER variants, and indeed the truncated transcripts would not be included in such an analysis (25, 39). A competitive amplification occurs amongst all exon-deleted or inserted ER variant transcripts, which depends on their initial relative expression, and the detection of bands corresponding to specific ER variants reflects the relative expression of these ER variant mRNA species within the samples. A survey of 100 breast tumours (20), showed that the most frequently expressed ER variants at a relatively high abundance were the exon 7-deleted variant, the exon 4-deleted variant, a variant deleted in both exons 3 and 4, a variant deleted in exons 2, 3 and 7, a variant deleted in both exons 4 and 7, a variant deleted in exons 2, 3 and 4, and a variant deleted from within exon 3 to within exon 7. Neither the exon 5-deleted nor the exon 3-deleted ER mRNAs were detected using this approach. Interestingly, preferential detection of some deleted variants was found to be associated with known prognostic markers in breast cancer (20).

In summary, data exist to support the hypothesis that altered expression of variant ER mRNA expression occurs during both breast tumorigenesis and breast cancer progression.

Expression of Variant or Mutant ER Proteins

It is unclear at this stage whether all or any of the ER-like transcripts so far identified are stably translated *in vivo*. It is certainly possible for many of them to be expressed at high levels from expression constructs transfected into mammalian, yeast and bacterial host cells. Furthermore, in some cases ER variant expression under these conditions has identified a putative function of the resulting variant protein (11, 13, 15). For example, exon 3 and exon 7-deleted variants may act as dominant negative regulators (inhibitors) of wild-type ER (11, 40) whereas exon 5-deleted ER has ligand-independent transcriptional activity (13, 43) (see discussion below).

More importantly, an ER-like protein consistent with that predicted to be encoded by the exon 5-deleted ER transcript has been found to be expressed naturally in some BT-20 human breast cancer cell lines (44). In addition, an immunoreactive 80 kDa ER-like protein has

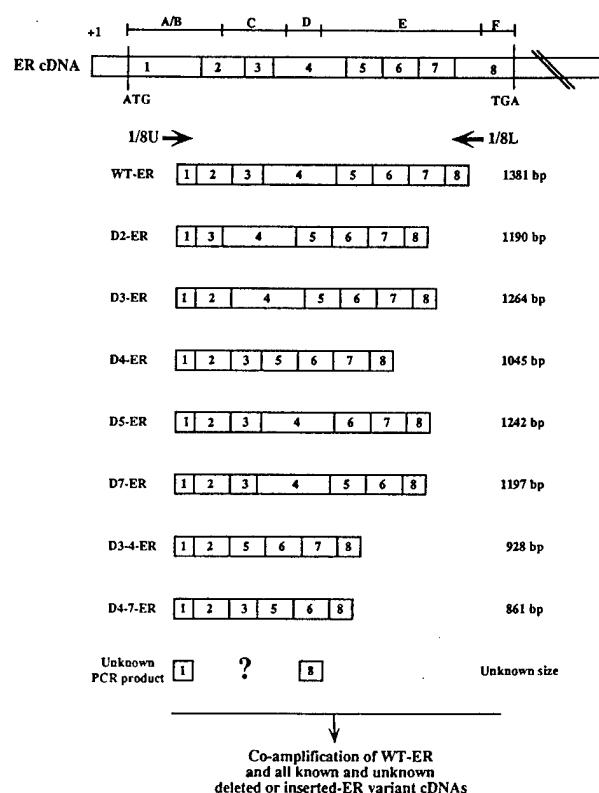


Figure 3. Schematic representation of wild-type oestrogen receptor (WT-ER) cDNA and primers allowing coamplification of most exon-deleted ER variants. 1/8U and 1/8L primers allow amplification of a 1381 bp fragment corresponding to WT-ER mRNA. Coamplification of all possible exon-deleted or inserted variants that contain exon 1 and 8 sequences can occur (20). Amplification of the previously described ER variant mRNAs deleted in exon 2 (D2-ER), exon 3 (D3-ER), exon 4 (D4-ER), exon 5 (D5-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER) and exons 4 and exon 7 (D4-7-ER) would generate 1190 bp, 1264 bp, 1045 bp, 1242 bp, 1197 bp, 928 bp, 1073 bp and 861 bp fragments, respectively.

been identified in an MCF-7 subclone (45). This protein corresponds to the predicted protein encoded by an ER-like transcript containing an exon 6 and 7 duplication, which was cloned from the same cell line (27). These data demonstrate the ability of some ER-like transcripts to be naturally translated into stable proteins, which can be detected by current methods, and suggest the likelihood of other ER-like transcripts being stably translated *in vivo* under natural conditions.

Other studies support the expression of variant or mutant ER-like proteins but their relationship to known variant or mutant ER mRNA remains unclear. Immunohistochemical staining with a polyclonal antibody was used previously to identify two types of apparently defective ER in human breast cancers (46); one that bound the nucleus in a ligand-independent fashion and one that could not bind to the nucleus even in the presence of ligand. Several other studies have identified ligand-binding forms of the ER that have both altered molecular mass (often truncated compared to the wild-type ER) and altered isoelectric points (47). The correlation of some of these ER-like proteins with biological parameters suggests that they may play a role in the ER signal transduction pathway (48). More recently, truncated DNA-binding forms of ER-like proteins have been identified in some human breast cancer biopsy samples (49). ER antibodies (Fig. 4) recognizing epitopes in the A/B and E domains of the wild-type receptor were found to detect these truncated ER-like proteins. An ER-like protein was identified in some ER+/PR- human breast tumours that formed complexes with an oligonucleotide containing an ERE in gel shift assays (15). The complex was supershifted by H226 and H222 antibodies but not by the D75 antibody recognizing a more C-terminal epitope (Fig. 4). Steroid hormone-induced mammary tumours in Grunder mice progress from hormone dependence to hormone independence following serial transplantation. This progression is associated with decreased expression of the 65 kDa ER protein and a marked increase in tamoxifen aziridine-bound, immunopurified 50 and 35 kDa proteins (50).

The relationship of any of these ER-like proteins that have been characterized in some human and mouse

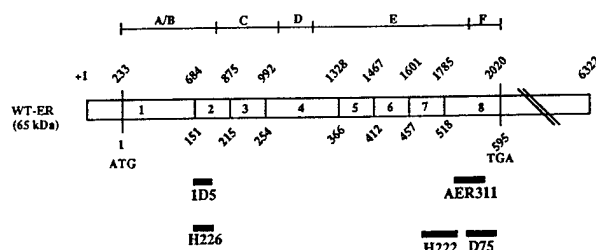


Figure 4. Approximate location of the epitopes recognized by the various oestrogen receptor antibodies (1D5, H226, H222, AER311, D75) referred to in this review. Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F). The numbering on the top of the cDNA refers to the nucleotide position as defined in (64) and the numbering below the cDNA refers to the amino acid positions as defined in (64).

tumour tissues to ER-like proteins that are potentially encoded by some of the previously characterized ER-like mRNAs is unclear and remains to be elucidated. Very few, if any, Western blotting analyses using differential antibody detection of ER-like proteins in human breast tumours have been reported. One study, where ER antibodies recognizing epitopes within the ligand-binding domain were used for Western blotting, identified larger than wild-type as well as smaller than wild-type ER immunoreactive proteins (49, 51). However, many of the known variant ER transcripts are likely to encode proteins around the size of heavy and light immunoglobulin chains. Immunoglobulin contamination of human breast tumours and immunoprecipitated complexes would probably interfere with Western blot analysis of such variant ER proteins (51).

More recently, a group of human breast tumours were analyzed immunohistochemically (52) for ER expression by using antibodies that recognize either an N-terminally localized epitope in the wild-type ER protein, or a C-terminally localized epitope in the wild-type ER protein (Fig. 4). It was found that the antibody recognizing the C-terminally localized epitope correlated better with the ligand-binding assays performed on adjacent tissues than did the antibody recognizing the N-terminally localized epitope. Additionally, although in many tumours the immunohistochemical results using each antibody showed good concordance, in some tumours the results were discordant, with the signal tending to be higher with the N-terminal antibody (53). Because many of the proteins predicted from variant ER mRNAs would be truncated at the C-terminus and would not contain the epitope recognized by the C-terminal antibody, one interpretation of these data would be that truncated variant ER proteins are more highly expressed in the discordant group of tumours. This hypothesis was tested by investigating the pattern and relative expression of variant ER mRNAs in the discordant and concordant groups of breast tumours. Several ER variant mRNAs that encode putative short ER-like proteins that would be recognized only by an N-terminal-targeted antibody were preferentially and more highly expressed in the discordant breast tumour group. These ER variants were: the clone 4-truncated ER mRNA; the exon 2, 3 plus 7-deleted ER mRNA; the exon 2, 3 plus 4-deleted ER mRNA; and the variant deleted within exon 3 to within exon 7 (53). The data suggest that the ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER measured by immunodetection assays using N- or C-terminal antibodies. Further, the data are consistent with the ability of ER variant mRNAs to be stably translated *in vivo* and therefore have a functional role or roles in ER signal transduction.

Structure and Function *In Vitro* and *In Vivo* of Putative Variant and Mutant ER-like Proteins

The data summarized in the previous section suggest the likelihood of some or all ER-like transcripts, being

stably translated *in vivo*. This provides a rationale for discussing the possible structure and function of the proteins predicted to be expressed from variant and/or mutant ER-like mRNAs.

1. Exon 7-deleted ER

Relative to all other deleted ER transcripts the exon 7-deleted ER variant appears to be the most abundant in human breast tissues (20). This transcript was first identified in T-47D human breast cancer cells (11) and was subsequently found in human breast tumour samples (15). The predicted protein encoded by this variant ER transcript is approximately 51 kDa (Fig. 1, Table 1), and is identical to the wild-type ER protein up to amino acid residue 456 and thereafter encodes 10 novel amino acids. The putative protein encoded by the exon 7-deleted transcript would therefore be truncated in the E domain, which includes the ligand binding, AF-2 and a strong dimerization domain of the wild-type receptor. The putative function of the protein encoded by the exon 7-deleted transcript is controversial. Wang and Miksicek (11) using HeLa cells found that it did not bind ERE DNA or have transcriptional activity of its own. Moreover, under these conditions the exon 7-deleted ER did not affect the activity of the wild-type ER. These data contrast with those obtained by Fuqua et al. (15), where, using a yeast expression system, the exon 7-deleted ER protein was found to inhibit wild-type ER activity. Furthermore, Fuqua et al. had originally isolated the exon 7-deleted ER variant mRNA from an ER+/PgR- breast tumour that contained an ER-like protein able to bind to DNA containing an ERE as determined by gel mobility shift analysis, but that interacted differentially with ER antibodies suggestive of an ER-like protein with a C-terminal truncation (15). The data of Fuqua et al. were consistent with the idea that over-expression of an exon 7-deleted ER protein could contribute functionally to the ER+/PR- breast tumour phenotype. The hypothesis was further supported by the observation that exon 7-deleted mRNA levels were significantly elevated in a group of human breast tumours that were ER+/PgR-/pS2- compared to those which were ER+/PgR+. Although exon 7-deleted mRNA was found in normal breast tissue, its expression was not significantly different in normal versus breast tumour tissue, although in the same tissue samples the levels of both the exon 5-deleted and the clone 4-truncated ER mRNAs were significantly higher in tumours compared to normal breast tissues (16). This latter study, in contrast to studies reported by Fuqua et al., did not find any significant relationship between exon 7-deleted ER mRNA level and PR status or tumour grade (16, 20).

The reported data suggest that the activity of the exon 7-deleted ER may vary in a cell-type and promoter-specific fashion. This in turn suggests that the background milieu may dictate the impact of variant ERs. So, although there are a few reports of altered exon 7-deleted ER expression, it is one of the most abundantly expressed variants in human breast tissues

and activity of this variant may depend on an altered cellular milieu.

2. Exon 5-deleted ER

The predicted protein from the exon 5-deleted ER mRNA is a truncated protein of approximately 50 kDa as a stop codon has been introduced after amino acid residue 371 (Fig. 1, Table 1). Its amino acid composition would be identical to the wild-type ER up to amino acid 366 followed by five novel amino acids, and consequently the majority of the ligand-binding domain of the wild-type ER will be missing.

Using a yeast expression system Fuqua et al. (13) showed that the exon 5-deleted ER displayed ligand-independent transcription from an ER-regulated reporter plasmid. Rea and Parker (54) confirmed this result in chicken embryo fibroblasts. However, when an exon 5-deleted ER expression vector was stably transfected into MCF-7 cells, it had no effect on an ERE-tk-luciferase reporter plasmid, it slightly increased transcription from an ERE₂TATA-CAT but not an ERE₁TATA-CAT reporter plasmid, and it had no effect on endogenous oestrogen-responsive genes such as pS2 and progesterone receptor. Neither did it result in the development of oestrogen independence and antioestrogen resistance in these cells. In contrast, a similar study by Fuqua and Wolf (55) showed that over-expression of the exon 5-deleted ER protein resulted in increased progesterone receptor levels in the absence of oestrogen, as well as oestrogen-independent growth and tamoxifen resistance. The reasons for the different results between the two groups are unclear, although differences in the original parent MCF-7 cells was suggested, in turn suggesting that other changes in addition to altered exon 5-deleted ER expression are required for hormonal progression in human breast cancer cells. This is not unreasonable as several mechanisms, either alone or in combination, may be responsible for such progression (10). For example, it is possible that the alteration of growth factors or their cognate receptors, some of which have been shown to result in ligand-independent activation of the wild-type ER through the N-terminal AF-1 domain (56, 57), may also be required in conjunction with altered expression of ER variants. Interestingly, Klotz et al. (58) identified a correlation between increased expression of the exon 5-deleted ER transcript relative to the wild-type ER and reduced responsiveness to oestrogen in MCF-7 stocks obtained from various laboratories in North America.

Measurement of the exon 5-deleted ER mRNA in clinical samples provides further insight into a possible role for this ER variant. The exon 5-deleted ER transcript was found to be present in normal human mammary tissue, but its level relative to the wild-type ER mRNA was significantly increased in breast tumour tissues (16). The exon 5-deleted ER transcript was first identified in a tumour that was ER-/PgR+, a finding consistent with the speculation concerning the ligand-independent activity of a protein encoded by this transcript (13). Using a specific RT-PCR approach, this transcript has been found to be more highly expressed,

and in some cases more abundant, than the wild-type ER mRNA, in ER-/PgR+ breast tumours (13, 42). However, when measured within a wide range of ER+/PR+ breast tumours, using a long-range RT-PCR approach (20), its relative expression with respect to all other deleted transcripts is low to undetectable (20, 59). Again using a specific RT-PCR analysis, Daffada et al. (42) found significantly higher levels of the exon 5-deleted ER transcript in those human breast tumours that were ER-/PgR+ or ER-/pS2+. However, while levels of the exon 5-deleted ER transcript are found to vary widely in human breast tumours, no significant differences in their levels were found between tamoxifen-resistant and tamoxifen-sensitive tumours (42). Furthermore, in a tamoxifen-resistant MCF-7 cell line the level of the exon 5-deleted transcripts was lower than the sensitive parent line (19) although differential expression of other ER variants was found. Presently no clear-cut correlation between exon 5-deleted ER expression and tamoxifen resistance is evident. However, this might be expected because there are multiple variants expressed in any one tumour and multiple mechanisms are likely to be involved in the development of tamoxifen resistance in particular and endocrine therapy resistance in general (10).

In conclusion, the putative activity of the protein encoded by the exon 5-deleted ER mRNA could contribute to the development of oestrogen independence and endocrine resistance in human breast cancer. Certainly correlations between the level of this transcript and apparently constitutively elevated oestrogen target gene expression would support this hypothesis. However, differences between the phenotypes generated by stable transfection studies and the lack of correlation of this transcript with tamoxifen-resistant breast tumours suggest that other factors are probably involved, either together with or independently of elevated exon 5-deleted ER expression. Although the naturally occurring exon 5-deleted ER mRNA and its putative cognate protein have been the most widely studied ER variants to date, we now know that multiple ER variants can be found in both normal and neoplastic breast tissues (16-18). Furthermore, using assays that allow the investigation of the relative expression of multiple ER variant mRNAs, it is apparent that the exon 5-deleted transcript represents one of the lower abundance variant transcripts in a wide range of human breast tumours, except perhaps in the ER-/PR+ phenotype.

3. Exon 4-deleted ER

The exon 4-deleted ER transcript has been found expressed in human breast cancer cell lines (12, 19, 59), human breast cancer tissue (17, 18, 20) and normal human breast (17, 18). This transcript contains an in-frame deletion and is predicted to encode a protein of approximately 54 kDa (Fig. 1, Table 1) which would be missing a strong nuclear localization domain and a portion of the E domain of the wild-type ER. When an expression vector was made for this variant, the

encoded protein did not bind oestradiol or an ERE, and had no transcriptional activity of its own nor any dominant negative activity against the wild-type ER (61, 62). Although these studies suggest that an exon 4-deleted ER is essentially inactive, negative results may reflect the promoter and cell types used in these studies. More recently, a correlation was found between the relatively increased expression of the exon 4-deleted ER mRNA with high PR expression and low grade, suggesting its correlation with some good prognostic features in human breast tumours (20). However, any functional role that this ER variant might have in this correlation is as yet unclear.

4. Exon 3-deleted ER

An exon 3-deleted ER transcript was initially identified in T-47D human breast cancer cells (11). A deletion of exon 3 from the wild-type ER transcript is in frame and generates a protein of approximately 61 kDa that lacks the second zinc finger of the wild-type ER DNA-binding motif. The function of the putative protein encoded by this transcript is controversial. When expression vectors for this protein were transfected into HeLa cells the exon 3-deleted protein demonstrated a dominant negative activity, inhibiting wild-type ER transcriptional activity, without any intrinsic transcriptional activity of its own. This variant ER, while unable to bind to an ERE in a gel mobility shift assay, inhibited the ability of the wild-type ER to bind to an ERE under the same conditions (11, 14). Preliminary data in which this variant was stably over-expressed in MCF-7 human breast cancer cells suggest that it has dominant negative activity in this model as well. Over-expressing cells were growth-inhibited by oestrogen, suggesting that this variant can inhibit the mitogenic effect of oestrogen in these cells (40). In contrast, in a yeast expression system this variant ER does not have transcriptional or dominant negative activity (63).

Again the data available in the literature concerning the potential activity of the exon 3-deleted ER suggest that variant activity as well as wild-type ER activity can depend on the gene promoter used and the cellular milieu. Interestingly, reported in abstract form is the observation that the level of the exon 3-deleted ER transcript is higher in normal mammary epithelia compared to breast tumours and tumour cell lines (40). This raises the interesting possibility that the expression of this variant may decrease with breast tumourigenesis and the exon 3-deleted ER may have an important role in the control of ER signalling and the control of breast epithelial cell growth. In a range of 100 breast tumours, using an approach that allowed the investigation of the relative expression of multiple ER-deleted mRNAs, the exon 3-deleted variant transcript was low to undetectable (20), while another study suggested that the level of this variant was similar in all ER+ breast tumours and was therefore unlikely to be involved in the evolution of the ER+/PgR- breast cancer phenotype in contrast to the exon 7-deleted variant (63). However, no compari-

son with normal human breast tissue was made in either of these two latter studies.

5. Exon 2-deleted ER

An ER-like transcript deleted in exon 2 sequences was first demonstrated in T-47D human breast cancer cells (11). Subsequently, it was identified in MCF-7 cells (19) and both normal (16–18) and neoplastic breast tissues (16–18). The exon 2-deleted transcript could encode a truncated protein of approximately 16 kDa missing the entire DNA- and ligand-binding domains (Fig. 1, Table 1). The protein would only encode the A/B region of the wild-type ER up to amino acid 151 with an additional novel amino acid residue. The protein encoded by this transcript displayed no transcriptional activity of its own, but exhibited a mild dominant negative activity when over-expressed at least 20-fold relative to the wild-type ER protein (11). This transcript was found to be over-expressed in a tamoxifen-resistant MCF-7 cell line compared to the parent MCF-7 cells, although other ER variant transcripts were also differentially expressed in these two cell lines (19). Although such data support a role for altered ER variant expression in hormone independence, the mechanism or mechanisms by which this is achieved is unknown.

6. Multiple-exon-deleted and Other Deleted ERs

Several multiple-exon-deleted ER transcripts have recently been identified in human breast cancer cells (19), and in both normal and neoplastic human breast tissues (16–18, 20). These include both double- and triple-exon deletions. Deletions of exons 4 and 7 from the one transcript have been described in human breast cancer cells (19) and human breast tissue (18). Furthermore, this transcript is frequently expressed at a relatively high level in a wide range of human breast tumours (20). An exon 4- and 7-deleted ER transcript is predicted to encode a protein of approximately 39 kDa (18) deleted in the hinge region, lacking a nuclear localization signal and significant portions of the ligand-binding and AF-2 domains. No studies reporting putative function have been published. Leygue et al. (16, 20) have identified transcripts deleted in exons 2 and 3, and transcripts deleted in exons 3 and 4 in human breast tissues. This latter transcript was also identified in human breast tissues by Gotteland et al. (17). The transcript is predicted to encode an inframe protein of approximately 49 kDa, lacking ER amino acid residues encoded on exons 3 and 4, i.e. amino acids 216–365 (Fig. 1, Table 1). This protein would be unable to bind to DNA, would be missing a nuclear localization signal and part of the hormone-binding domain. Interestingly, the relative expression of this transcript is increased markedly in human breast cancer cells that have become oestrogen independent (A. Coutts, E. Leygue and L. Murphy, unpublished observation). No data are available with regard to the potential function of this protein in human breast cells, although the protein encoded by this transcript has no transcriptional or

dominant negative activity in a rat aortic smooth muscle cell line model (61).

Triple-exon-deleted ER transcripts have been observed in MCF-7 human breast cancer cells (18) and in human breast cancer tissue (20). MCF-7 cells contain ER transcripts deleted in exons 3, 4 and 5 as well as transcripts deleted in exons 5, 6 and 7 (18). Leygue et al. (20) recently identified in human breast cancer tissues ER transcripts deleted in exons 2, 3 and 4 and exons 2, 3 and 7. No functional studies have been carried out on the proteins encoded by such transcripts; however both these transcripts were frequently detected at a relatively high level of expression in a wide range of human breast tumours (20). In addition, the detection of the exon 2, 3 and 4-deleted transcript was significantly correlated with high-grade tumours (20).

Deletions that are not exact exon deletions have also been described. Grahame et al. (21) identified in T-47D cells an ER-like transcript deleted of 462 bases from within exon 4 to within exon 5. This predicts for a putative protein containing 442 amino acids with an in-frame deletion of 153 amino acids of the wild-type ER protein (Fig. 1, Table 1). The predicted protein is deleted from the end of the DNA-binding domain to mid-ligand-binding domain. This same group observed an ER transcript deleted in a G residue (nucleotide 1463) (numbered according to (64) of wild-type ER sequence) at amino acid residue 411 in the hormone-binding domain of the ER. This resulted in a frame shift so that a truncated protein is encoded (Table 2). The predicted protein is identical to the wild-type ER up to amino acid residue 410, followed by seven novel amino acids. The protein would have an intact DNA-binding domain and hinge region but would be truncated in the ligand-binding domain. Similarly, Karnik et al. (22) identified an ER-like transcript in a tamoxifen-resistant metastatic human breast tumour that was deleted in a T residue in exon 6. This would generate a frame shift resulting in a protein identical to the wild-type ER up to amino acid residue 433 followed by five novel amino acids (Table 2). This protein is probably defective in its ligand-binding and AF-2 activities. Daffada and Dowsett (23) described a novel splice variant of the ER mRNA in normal human endometrial tissue and breast cancers. This variant consists of a deletion within exon 4 sequences to within exon 7 sequences. This variant is out of frame, is identical to the ER up to amino acid residue 277 and thereafter encodes another 32 novel amino acids (Fig. 1, Table 1). The predicted protein would lack a large part of the ligand-binding domain and the AF-2 domain, but would contain the AF-1 domain, the DNA-binding domain and the nuclear localization signal of the wild-type ER. Leygue et al. (20) have identified ER transcripts in a wide range of human breast cancer samples, which are deleted from within exon 3 to within exon 7. This transcript was frequently detected in breast tumours, and in particular its expression was significantly correlated to tumours with very high levels of wild-type ER up to amino acid residue 232 and would then encode a further 18 novel amino acids (Fig. 1, Table 1). However, the protein lacks some of the

DNA-binding domain, all of the ligand-binding domain, and the AF-2 function.

7. Truncated ER mRNAs

The truncated ER-like transcripts (24, 25), which consist of various combinations of exons 1, 2 and 3 of the normal ER mRNA followed by sequences that are not found in the wild-type ER mRNA, were initially identified on Northern blots as abundantly expressed smaller-sized ER transcripts in some human breast cancer biopsy samples. This analysis identified them as abundant or more abundant than the wild-type transcript in some human breast cancer samples (24). Subsequently, several of the cognate cDNAs for these truncated transcripts were cloned and characterized, and found to contain authentic polyadenylation signals and poly A tails. The clone 24- and clone 5-truncated transcripts were found in only one breast tumour but the clone 4-truncated ER mRNA was found to be expressed in a wide range of breast tumours (25). Clone 5, however, consisted of exon 1 and 3 followed by ER unrelated sequences, and therefore is an example of a mixed exon-deleted and truncated transcript. Clone 4 consists of exons 1 and 2 of the wild-type ER mRNA followed by LINE-1 sequences (25). It could encode a protein of approximately 24 kDa, which would be identical to amino acid residues 1–214 of the wild-type human ER protein (25) (Fig. 1, Table 1) and thereafter encodes another six novel amino acids that are not found in the wild-type human ER. If the clone 4 mRNA were translated it would encode a protein that is identical to the A/B region and the first 'zinc finger' of the normal ER protein, but would be missing the second 'zinc finger', nuclear localization domains and the E domain of the normal ER protein (4). However, the protein had no transcriptional or dominant negative activity in transient transfection assays (25). Support for a role for this variant in human breast cancer progression comes from data that show that the relative level of expression of this variant is significantly elevated in breast tumours versus normal mammary gland (39) and that the relative level of expression of this variant is significantly elevated in breast tumours with characteristics of poor prognosis and endocrine resistance versus those with characteristics of good prognosis and endocrine sensitivity (41).

8. Point Mutations in the ER

Several point mutations have been identified in the human ER. The first of them was a G-to-C mutation (30–32), which was a silent polymorphism at nucleotide 261 (using the numbering presented in (64)). Although this is a silent polymorphism, the B-region variant allele (B') of the ER has been correlated with decreased levels of oestrogen binding in human breast cancers (65), increased history of spontaneous abortion in women with ER-positive breast cancer (66), increased height in women (67) and possibly increased prevalence of hypertension (68).

A C-to-T transition at codon 157 in exon 2 of the human ER appears to be the cause of oestrogen resistance in a man (36). The mutation results in a premature stop codon so that a protein truncated within exon 2 would be formed, encoding only the A/B region and missing both zinc fingers of the DNA-binding domain as well as the entire hormone-binding domain. This is the first identified disease causing mutation in the human ER. Interestingly, this study demonstrated that disruption of the ER gene need not be lethal in humans and identified the importance of oestrogen in bone maturation and mineralization in men as well as women (36).

Point mutations have been identified in the ER in some breast cancers. A silent polymorphism (T-to-C) at serine 10 has been identified by at least two independent groups (25, 29). A leucine to proline substitution at amino acid residue 296 has been identified in two breast tumours (33); however, the functional significance of this is unknown. A C-to-G change that is a silent polymorphism at proline 325 (33) has also been observed. Karnik et al. (22) identified an A-to-T nucleotide change in one breast tumour, which would alter Glu 352 to Val as well as several silent polymorphisms (C-to-T in Gly 276; G-to-T in Lys 472; C-to-G at Ala 505; T-to-C at His 577). However, none of these was frequently observed and none correlated with tamoxifen sensitivity or resistance in this group of human breast tumours. The point mutation changing Gly 400 to Val that was introduced into the human ER cDNA, as a cloning artifact, was shown to alter the receptor's affinity for oestrogen under certain conditions (69), as well as to enhance the oestrogenic activity of 4-hydroxy-tamoxifen in stable ER transfectants of MDA-MB-231 human breast cancer cells (64). Moreover, the ER from an MCF-7 tumour line, which was stimulated by tamoxifen, contains a point mutation so that Asp 351 was changed to a Tyr residue (34). This mutant ER was subsequently shown to result in increased oestrogenicity of a tamoxifen analog (35).

9. Insertions in the ER

ER mRNAs containing inserted sequences have been identified in approximately 9% of human breast tumours (26). Three types of inserted sequences were identified: one in which a complete duplication of exon 6 was found, one in which a complete duplication of exons 3 and 4 was found, and one in which 69 novel nucleotides had been inserted between the exon 5 and 6 sequences of the normal ER mRNA. The functional significance of such alterations is as yet unclear. However, the exon 6-duplicated ER-like mRNA predicts a protein of 51 kDa identical to the wild-type ER but would be truncated in the mid-E domain. Deletion and site-directed mutagenesis data suggest that such a protein would not bind oestradiol (2, 4, 71–74). Further, an important dimerization interface and the ligand-dependent AF-2 activity would be missing in the protein predicted from the exon 6-duplicated ER-like mRNA. However, a weaker constitutively active dimerization domain present in the DNA-binding domain, as well as the constitutive nuclear localization signal present in

exon 4 of the wild-type ER (75) and the ligand-independent AF-1 activity in the A/B domain would still be present (5). Preliminary data suggest that the protein encoded by this transcript has no ability to bind oestradiol and has little, if any, transcriptional activity using a classical ERE reporter gene construct (D. Douglas and L. Murphy, unpublished observations).

The predicted protein from the exon 3 and 4-duplicated ER transcript is around 82 kDa (Fig. 2, Table 1). It is identical to the wild-type ER protein up to amino acid residue 366, followed by another 380 amino acid residues encoded by exons 3 to 8. Therefore the amino acid residues encoded by exons 3 and 4 are completely duplicated. This protein would contain the AF-1 domain located in the A/B region of the wild-type ER, as well as the DNA-binding and dimerization domains and the constitutive nuclear localization signal of the wild-type ER protein, but would then have a third zinc finger encoded by exon 3, another nuclear localization signal followed by the normal E-domain containing ligand binding, AF-2 and dimerization functions. The presence of the extra ER residues from exons 3 and 4 would probably result in an altered structure of the protein, which may affect several of its normal functions. Preliminary data suggest that the protein encoded by this transcript has reduced oestradiol-binding activity and reduced, but still detectable, ligand-activated transcriptional activity (D. Douglas and L. Murphy, unpublished observations).

The unique 69-bp insertion is in-frame and codes for 23 novel amino acids inserted between residues 412 and 413 of the normal ER protein (Fig. 2, Table 1). This would result in a protein of approximately 69 kDa. While all residues of the wild-type ER are present in this protein the inserted sequence may cause an alteration of the structure in the E domain of this protein, so that some alteration or disruption of function may occur. Preliminary data suggest the protein encoded by this transcript has reduced oestradiol-binding activity and little, if any, transcriptional activity of its own (D. Douglas and L. Murphy, unpublished observations).

Interestingly, the identification of an immunoreactive ER-like protein of 80 kDa was recently reported in an MCF-7 subclone, which was oestrogen independent with respect to growth (45). The transcript possibly corresponding to this protein appeared to contain a precise duplication of both exons 6 and 7 (Table 1, Fig. 2). Also, an abnormal ER-like transcript was cloned from T-47D_{co} cells, which contained an insertion of approximately 130 nucleotides into exon 5 sequences (21). The inserted sequences displayed sequence similarity to the human *alu* family of repetitive sequences (21). The same group identified another mutant ER transcript in T-47D_{co} cells, in which two T residues were inserted in exon 3 resulting in a frame shift, changing amino acid 250 from methionine to an isoleucine, followed by a stop codon (21). The predicted protein would be truncated just beyond the last cysteine of the second zinc finger, with no hinge or ligand-binding domains (Table 2). Although no DNA binding/gel retardation analysis for this predicted protein was observed, the protein displayed weak constitutive transcriptional

activity, and higher concentrations had weak inhibitory activity when expressed together with the wild-type ER (76). In addition, some small insertions (1-3 nucleotides) have been described in the ER mRNA of some breast cancer biopsy samples (22, 29, 33) (Table 2). The frequency and significance of these are not known.

Conclusions and Unanswered Questions

There is a large amount of molecular evidence supporting the existence of variant and mutant ER proteins. While this evidence is derived mainly from characterization of mRNA species, data are now accumulating to suggest that the stable translation of ER variant mRNAs occurs at least in some human breast cancer tissues. This, in turn, suggests that any future examination of ER signal transduction and/or measurement of ER protein must take into account variant ER expression. The possible functions of variant ER proteins, either physiological or pathological, remain unclear, although correlative studies tend to support a role or roles for some ER variants in breast tumorigenesis and breast cancer progression. However, future speculation concerning these issues must take into account the presence of multiple ER variants in any one breast tissue sample, as well as the relative expression of each variant with respect to others, which can be altered in different groups of breast tumours, as discussed above. Furthermore, there are data that support the possibility that the pattern of ER variant expression can differ amongst different normal oestrogen target tissues (23), suggesting a possible role in the tissue-specific differences of ER signal transduction. These differences also dictate that analysis of putative function of any individual ER variant must also consider the cellular context as well as the promoter used to assess transcriptional function. This becomes increasingly important in the light of recent studies where novel oestrogen-responsive DNA sequences have been characterized, which remain quite distinct in structure-function activity and presumably mechanism from that classically determined using ERE sequences from the vitellogenin promoter (77-80). The recent cloning of a new ER, ER-beta (81, 82), with an overlapping but distinct pattern of tissue expression to the classical ER-alpha, also begs the question of whether the two ERs can interact and how the variant receptor forms may affect either or both signal transduction pathways.

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APPENDIX 2

Leygue E, Dotzlaw H, Lu B, Watson PH and Murphy LC

Estrogen receptor beta: a review.

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ESTROGEN RECEPTOR BETA: A REVIEW

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ABSTRACT

A single receptor, ER- α , was thought to mediate estrogen and anti-estrogen action in estrogen target tissues. Recently, a second estrogen receptor, known as ER- β , has been identified in several known (ovary, breast, bone) as well as "less conventional" (lung, heart, kidney) estrogen target tissues in human, mouse and rat. ER- β , which is also a member of the steroid/thyroid/retinoic acid receptor super-family, shares a similar structural and functional composition to ER- α and is able to activate the transcription of target genes through identical estrogen responsive elements. The observation of differential activation of ER- α and ER- β by anti-estrogens together with their ability to form hetero-dimers, suggests however that these two receptors might have different roles and that putative cross-talk of their signaling pathways might exist. Estrogen mechanism of action in any given tissue should therefore be re-evaluated. The purpose of this mini-review is to summarize the data published to date and to discuss the possible implications of the expression of ER- β in human breast cancer.

INTRODUCTION

Estrogens, that are known to regulate the growth and the development of reproductive female organs, also play key roles in other target tissues such bone, central nervous system and cardiovascular system. Estrogen effects were thought to be mainly mediated through a previously cloned and well characterized receptor, now referred to as estrogen receptor alpha (ER- α). ER- α , which belongs to the steroid-thyroid-retinoic acid receptor superfamily [1], was cloned in 1985 from a human breast tumor cell line cDNA library [2]. Like other members of this family, ER- α can be divided into several structural and functional domains (A-F) depicted in Figure 1 [3]. The A/B region of the receptor is involved in trans-activating function AF-1, whereas the C region contains the DNA-binding domain and the E region is implicated in hormone binding and another trans-activating function, AF-2. Upon ligand-binding, ER- α dissociates from a protein complex containing heat-shock proteins such as hsp 70 and hsp 90 to dimerize, and the resulting dimer binds to DNA at specific sequences called estrogen responsive elements (ERE) located upstream of the target gene [4]. Following interactions of the ER- α homo-dimer bound to the DNA and accessory co-activator proteins the transcription of such genes is eventually modified. Upon ligand binding, ER- α is also able to mediate the

transcription of AP1 regulated genes [5]. The ability of anti-estrogens such ICI-164,384, tamoxifen or raloxifene to bind ER- α and to modify its DNA-binding properties and its interactions with accessory proteins and ultimately its trans-activating activities, underlies their efficacy as endocrine therapies in breast cancer. In 1995, Kuiper et al. [6], isolated from a rat prostate cDNA library a 2.6 kb cDNA which encoded a molecule with strong sequence similarities to the DNA-binding domain (95%) and the hormone binding domain (60%) of ER- α (Figure 1). The discovery of this second estrogen receptor, called estrogen receptor beta (ER- β), led to the need to fully re-evaluate the molecular mechanisms of estrogen signal transduction in target tissues.

PRIMARY STRUCTURE AND VARIANT FORMS OF ER- β

Since the first report of the cloning of ER- β cDNA, that occurred more than two years ago, several groups have published (or submitted to Genbank) sequences of ER- β related molecules. However, the primary structure of ER- β still remains uncertain [7]. In order to understand the reasons behind the discrepancies observed, it is important to review the different cloning strategies used to identify ER- β related sequences.

Rat

Kuiper et al., looking for novel nuclear receptors, used degenerate primers, the sequences of which were based upon conserved DNA- and ligand-binding domains of nuclear receptors, to amplify rat prostate cDNAs [6]. They obtained a PCR product which when sequenced presented strong sequence similarities (65%) to the rat ER- α cDNA. Using this fragment as a probe, they isolated from a rat prostate cDNA library a 2.6 kb long cDNA which could encode a protein, initially called clone 29 protein, shown in Figure 2. This protein has strong sequence similarities with the ER- α DNA-binding and ligand-binding domains and the authors called this protein ER- β , to distinguish it from the previously identified estrogen receptor. Since then, several alternatively spliced forms of ER- β mRNA have been described and we will therefore refer to the protein encoded by clone 29 cDNA as rER- β 1 (for rat ER beta 1). Because of the presence of an in-frame stop codon upstream of the coding sequence obtained, it was assumed that the 2.6 kb rat cDNA encoded the full length rER- β 1 protein. The calculated molecular mass of the 485 amino acids encoded by this cDNA is 54.2 kDa. In vitro translated rER- β 1 protein migrated as a doublet on SDS/PAGE with an apparent

molecular mass of 61 kDa. The presence of a doublet was explained by Kuiper et al. as the possible result of the use of two different initiating codons for protein synthesis, but no mention was made regarding the discrepancy between the calculated and the observed molecular mass. Subsequently, most other rat ER- β sequences were obtained using reverse-transcription (RT), and polymerase chain reaction (PCR) using primers spanning the initial published coding sequence. Therefore, most of them share the same initiating methionine codon and the last glutamine codon of rER- β 1 (see Figure 2). In 1997, an inserted variant form of ER- β , referred to as rER- β 2, was identified by Chu et al. after RT-PCR amplification of rat ovary cDNA [8]. The existence of such a variant was subsequently confirmed by other groups [9, 10]. This variant consists of an insertion of 54 bp between exon 5 and exon 6 of ER- β . One should note here that as underlined later in the text, the exon/intron structure of ER- β gene established by Enmark et al. [11] is similar to that previously shown for ER- α [12]. This 54 nucleotide insertion is in frame and therefore the inserted transcript will encode an extra 18 amino acids within the ligand-binding domain of the molecule (Figure 2). Variant forms of rER- β 1 and rER- β 2 deleted in exon 3 were also described and referred to as rER- β 1 Δ 3 and rER- β 2 Δ 3, respectively [10]. This in frame deletion of exon 3 would result in the elimination of the second zinc finger of the DNA-binding domain of the receptor (Figure 2). In 1998, Aldridge et al. submitted to Genbank (accession number AJ002602) an ER- β sequence obtained from a cDNA isolated by RT-PCR from rat prostate cDNA. These authors, in contrast to the previously mentioned studies, used an upper primer recognizing sequences upstream of the putative in frame upstream stop codon observed in rER- β 1 cDNA. The 1650 bp long cDNA they obtained corresponds to the sequence between nucleotide 226 to 1874 of rER- β 1 except for 6 differences. These differences consisted of an additional C residue between C319 and T320, an A instead of T at position 496, a G instead of C at position 729, a C instead of T at position 774, a C instead of T at position 1034, and a C instead of T at position 1794. The extra nucleotide observed between nucleotides 319 and 320 of rER- β 1 sequence, alters the reading frame, suppresses the previously observed in frame upstream stop codon and results in this new ER- β sequence encoding an extra stretch of 64 amino acids upstream of the rER- β 1 protein sequence (Figure 2). The resulting 549 amino acid long protein, rER- β 1long, has a calculated molecular mass of 61.3 kDa. The nucleotide change at position 1034 does not affect the primary structure of the protein, but changes at other positions modify amino acid composition: glutamine, alanine, proline and proline are observed at position 27,

105, 120 and 450 instead of leucine, proline, serine and serine, respectively (See Figure 2). Interestingly, sequences published by Maruyama et al. [9] and Petersen et al. [10] also contained two of these amino-acids changes, at position 27 and 105. One should note that Genbank sequence AB012721 submitted by Maruyama et al. does not contain these modifications suggesting that these authors isolated two slightly different rER- β 2 isoforms [9]. These slight amino-acid differences, that may result from the cloning strategies used or the tissue studied should be noted since previously, a single amino-acid modification within the first ER- α sequence published was later shown to have a functional effect. The alteration caused an apparent destabilization of this receptor and a modification of its affinity for 17- β -estradiol (E2) [13]. The question of whether or not rER- β amino-acid changes affect protein function remains to be addressed.

Mouse

Tremblay et al. used a combination of PCR and cDNA screening to obtain the first "full length" mouse ER- β , mER- β 1 [14]. These authors using degenerate primers specific for the ligand-binding domain of rER- β 1 amplified a 550 bp fragment from mouse ovarian cDNA, that had strong sequence similarities to the rER- β 1 sequence. Using this fragment as a probe, they isolated from a mouse cDNA library 3 clones, the sequences of which started in the ligand-binding domain of the molecule and contained a poly-A tail. Using a downstream primer specific for their "new" 3' mouse sequence and an upstream primer spanning the first 21 bases of the 5'UTR of rER- β 1 and ending with the putative initiator methionine codon, Tremblay et al. eventually obtained the sequence encoding the "full length" mER- β 1 (Figure 3). This sequence was later confirmed by Petterson et al. [16] even though some nucleotide variations which modified amino acid sequence were observed. Alanine, threonine, asparagine, aspartic acid, histidine, arginine and glycine were observed at position 2, 97, 155, 333, 367, 400 and 466 instead of threonine, alanine, serine, glycine, proline, glycine and glutamic acid, respectively (Figure 3). The change at position 2 results directly from the sequence of the primer used by Petterson et al. to amplify the 5'-extremity of the cDNA, that encoded this amino acid change. Two sequences, recently submitted to Genbank, were both obtained by PCR amplification of mouse ovarian cDNA. They revealed that the N-terminal extremity of the mouse ER- β could be longer than previously shown. These two isoforms, mER- β 1med and mER- β 1long form, would encode a protein containing, compared to mER- β 1, 45 and 64 additional N-terminal amino acids, respectively (Figure 3). The mER- β 1med isoform would encode a protein starting at a methionine codon corresponding

to the methionine 20 of rER- β 1long, whereas the mER- β 1long form would encode a protein starting at a methionine corresponding to the first methionine of rER- β 1long. Similar to what was observed in the rat, an insertional variant ER- β mRNA containing an extra-stretch of 54 nucleotides at the junction of exon 5 and 6 has been described in the mouse [15]. This cDNA could therefore encode a protein, mER- β 2, identical to mER- β 1, except for the presence of 18 additional amino acids within the ligand-binding domain (Figure 3). Variant mRNAs deleted in exon 5 (mER- β 1 Δ 5), exon 6 (mER- β 1 Δ 6), and exon 5+6 (mER- β 1 Δ 5-6) have also been reported [15]. The deletion of exon 5 and of exon 6 separately leads to a shift in the open reading frame and the putative encoded proteins are therefore missing all of the C-terminal region of mER- β 1 (Figure 3). In contrast, the double deletion "exon 5 + exon 6" does not change the coding reading frame and the encoded protein will be deleted in 91 amino acids within the ligand-binding domain/AF2 region (Figure 3). Because these variant forms of mER- β have been observed using targeted PCR (i.e performed using primers spanning only a small portion of the mER- β 1 cDNA), no information is available to date to determine whether these deleted forms correspond to mER- β 1, mER- β 1med or mER- β 1long (Figure 3). Similarly, the partial 3' sequence of mER- β 2 and mER- β 1 Δ 5-6 cDNA does not allow the unequivocal determination of the sequence of the C-terminal extremity of the putative proteins encoded by these variants (Figure 3).

Human

In 1996, Mosselman et al. used a similar approach to that of Kuiper et al. to screen a human testis cDNA library. They identified two cDNAs encoding a protein with strong sequence homology to hER- α [17]. Interestingly, the sequence similarity observed stopped in both clones at the exact junction between sequences encoded by exon 7 and exon 8 as determined by analogy to hER- α . This observation led the authors to conclude that their cDNAs represented incompletely spliced transcripts. They therefore used RACE PCR amplification of testis cDNA to obtain the 3'terminal extremity of their cDNA. The resulting cDNA could encode the protein depicted in Figure 4. As observed for the rat, the comparison of the amino acid sequence of hER- β with hER- α showed a high conservation of the DNA-binding domain (96%) and of the ligand-binding domain (58%). However, the absence of an inframe stop codon upstream of the first Met suggested that the 5'coding extremity of the cDNA might be incomplete. In 1997, Enmark et al., using probes corresponding to regions encoding the N-terminal and hinge domains of rER- β 1 isolated several partial

clones of hER- β from human ovarian and prostatic cDNA libraries [11]. The first 45 and the last 59 amino acids of the sequence were obtained by PCR amplification of human ovary cDNA, using primers derived from rER- β 1. These clones were then joined by PCR amplification and restriction enzyme digestion. The sequence eventually obtained could encode a protein (hER- β 1) almost identical to that encoded by the Mosselman cDNA, except for the presence of 8 additional N-terminal amino acids, homologous to rER- β 1 sequences (Figure 4). In 1998, Ogawa et al. [19] screened a testis cDNA library with probes corresponding to the DNA-binding domain of rER- α , and identified a hER- β -like clone that contained extra 5' sequences in addition to the hER- β 1 sequence. This sequence could encode a protein with N-terminal amino acid sequences highly similar to the N-terminal sequence of mER- β 1med. Here again, PCR was necessary to obtain a full length cDNA. This amplification was performed using primers corresponding to the 5'-sequences of the new clone and to the previously published 3' extremity. The protein encoded by this "full length" cDNA is presented in Figure 4 (hER- β 1long). This 530 amino acid protein has a calculated molecular mass of 59.2 kDa. In vitro translated hER- β 1long migrated as a doublet with an apparent size of 60 and 57 kDa suggesting the use of two different initiating codons. More recently, Moore et al. screened a single stranded human testis cDNA library with biotinylated-hER- β 1 oligonucleotides and isolated, in addition to hER- β 1long, two full length variant ER- β cDNAs, which could encode hER- β 2 and hER- β 3 [20]. These ER- β isoforms are identical to hER- β 1long protein, except that they differ in their C-terminal extremities (Figure 4). In particular, they do not contain the region encoded by exon 8 of the hER- β 1 cDNA sequence. The 495 amino acid hER- β 2 and the 513 amino acids hER- β 3 proteins are missing a part of the ligand-binding domain of the hER- β 1 molecule and are therefore smaller, with calculated molecular masses of 55.5 and 57.5 kDa, respectively. The hER- β 2 isoform was also recently cloned by Ogawa et al. [21]. These authors have named their isoform ERbetacx and it is identical to hER- β 2. It is important to note that the suffix "- β 2" describes in the human species a particular truncated variant whereas in the rodents it refers to an inserted variant. There is no evidence of an equivalent to the rodent inserted ER- β 2 variant in human tissues [15]. Using PCR, Moore et al. [20] isolated partial cDNA sequences encoding hER- β 4 and hER- β 5 (Figure 4). These cDNAs share the sequence encoded by exon 7, but differ in their 3'extremity and do not contain exon 8 sequences. The putative proteins encoded by these variant cDNAs will therefore be missing a part of the ligand-binding domain of the hER- β 1 molecule. One should note that because these cDNA

isoforms have been observed using RT-PCR amplification of only a limited region of the molecule, no information is available regarding the putative N-terminal sequence of the encoded proteins (i.e whether they correspond to hER- β 1long or to hER- β 1short). It is unclear at present if ER- β variants analogous to the C-terminally truncated hER- β 2, hER- β 3, hER- β 4 and hER- β 5 exist in the rodent. In 1997, using primers recognizing sequences in exons 1 and 8 of hER- β 1, we have successfully amplified from human breast tissue RNA, a variant form of hER- β cDNA deleted in both exon 5 and exon 6 [22]. This in frame deleted cDNA could encode a hER- β like molecule, referred to as hER- β 1 Δ 5-6 (Figure 4), which is deleted in 91 amino acids within the region containing the hormone binding domain and the trans-activating function 2 of the molecule. The existence of this variant as well as others, deleted in exon 5 (hER- β 1 Δ 5) or exon 6 (hER- β 1 Δ 6) was later confirmed [15, 18, Genbank AF074599]. These variant forms of hER- β mRNA have been observed using targeted PCR and only partial cDNA sequences are known. No information is available to date to determine whether these deleted forms correspond to hER- β 1short or hER- β 1long (Figure 4). Similarly, the C-terminal extremity of the putative hER- β 1 Δ 5-6 protein remains to be determined (Figure 4).

It is interesting to note that amongst all ER- β sequences described to date, only four have been obtained by direct cDNA subcloning: rER- β 1 [6], hER- β 1long, hER- β 2 and hER- β 3 [20]. Since rER- β 1 sequence was the first one published, it is considered the "wild-type" molecule. Most of the cDNAs isolated directly from cDNA libraries encoded partially truncated ER- β 1 molecules that were presumed incomplete. The apparent high frequency of detection of such partial sequences raises the question of what molecule represents the major ER- β form in a given tissue. In other words, given the fact that hER- β 1 and mER- β 1 required the use of PCR to be isolated, should they be considered as the "wild-type" molecule in all tissues? Similarly, do longer and/or different variant forms, still unidentified, exist?

PRIMARY STRUCTURE AND CHROMOSOMAL LOCALIZATION OF THE ER- β GENE

As mentioned above, the primary structure of the ER- β gene was established by Enmark et al. for the mouse and the human [11]. Similar to the ER- α gene (Figure 5), the ER- β gene is composed of 8 exons. The

ER- β gene exon/intron structure appears conserved between the two species and corresponds with the exon/intron structure observed for ER- α . The ER- β gene however differs from hER- α gene by its length, 40kb versus >140kb. The chromosomal localization of the ER- β gene has been established for the human (14q22-24 [11]) and the mouse (chromosome 12 [14]). Even though the structure of the rat ER- β gene has not been formally established to date, the existence of similar splice variants (such as rER- β 2 or rER- β 1 Δ 3) strongly suggests that the rat ER- β gene will share a similar structure to that observed in mouse and human.

FUNCTIONAL FEATURES

Most of the functional studies published so far, have been performed using the shorter molecules like rER- β 1, rER- β 2, mER- β 1 or hER- β 1short. As outlined above, these receptors may be missing the N-terminal extremity of the protein. This region, by homology to the N-terminal extremity of ER- α , could be involved in the trans-activating-function 1 of the protein. The AF1 domain is known to be partially responsible for the agonistic effect of anti-estrogens such as tamoxifen on ER- α . To our knowledge, no study investigating the putative functional differences between long and short ER- β forms has yet been published. The main functional features of the rat, the mouse and the human ER- β molecules have been summarized in Table 1.

Rat

In vitro and in vivo translated rER- β 1 protein was shown to bind 17 β -estradiol with K_d values around 0.4 nM [6, 9, 10]. Several other estrogenic substances such as diethylstilbestrol (DES), estriol or estrone are also able to bind rER- β 1 [6, 10, 23]. In contrast, other nuclear receptor ligands such as testosterone, progesterone or corticosterone are unable to bind to rER- β 1 [10, 23]. Kuiper et al. compared the relative affinity of numerous ligands for hER- α and rER- β 1 by ligand competition experiments and concluded that rER- β 1 had a ligand-binding spectrum which was overall similar to that of hER- α [23]. However, some compounds such 17 α -estradiol or moxestrol had a higher relative affinity for hER- α than for rER- β 1, whereas others, such as 4-hydroxy-tamoxifen (4-OH-Tam) and ICI164,384, had a higher relative affinity for hER- β . Comparing rER- β 1 and hER- α ligand-binding domains (LBDs) recombinantly expressed in E.coli, Witkowska et al. found a close structural relationship between E2-bound rER- β LBD and hER- α LBD

complexes [24]. These authors also suggested that while no significant differences exist between the response of hER- α and rER- β 1 LBDs to 4-OH-Tam and ICI164,384, some compounds such 16 α -bromo-estradiol and genistein showed selective interactions with hER- α and rER- β 1 LBDs, respectively. Gel mobility shift assays demonstrated the ability of rER- β 1 homo-dimers to bind a consensus ERE, even though the binding affinity observed was lower than that seen for rER- α homo-dimers [10]. Co-transfection experiments using an ERE-driven reporter gene and a rER- β 1 expression vector revealed a stimulatory effect of 17- β -estradiol [3, 9, 10, 25]. Since these assays were performed in several different cell systems (CHO, HeLa, Cos and 293S cells), this effect appears to be cell type independent. Kuiper et al. also showed that tamoxifen acts as an antagonist of E2 action in CHO cells [6]. Interestingly, the activity of rER- β 1 via AP1-driven reporter genes differs significantly to that observed for ER- α . Paech et al. [25] demonstrated that whereas estrogens such E2 or DES stimulated the transcription of such genes when bound to ER- α , they inhibited transcription when associated with rER- β 1. Moreover, while anti-estrogens such tamoxifen, ICI164,384 or raloxifene do not activate ER- α , they induce the transcription of AP1-regulated genes when bound to rER- β 1.

The rER- β 1 was shown to form hetero-dimers with rER- α and rER- β 2 [9, 10], and the formation of rER- β 1/rER- α hetero-dimers was favoured over rER- β 1/rER- β 1 and rER- α /rER- α homo-dimer formation in the presence of E2 [10]. The ability of ER- α and ER- β to hetero-dimerize adds an important layer of complexity to the mechanisms of estrogen signal transduction. Maruyama et al. showed that, in contrast to rER- β 1, rER- β 2 was unable to bind E2 [9]. However, Petersen et al. found that rER- β 2 can bind E2 but with a markedly reduced affinity (K_d value of 5.1 nM) [10]. Both of these studies showed that rER- β 2 was able to bind a consensus ERE. Whereas Petersen et al. showed that in the presence of 10nM E2, rER- β 2 could activate the transcription of an ERE-reporter gene, Maruyama et al. found no activation of an ERE reporter gene under similar conditions. Using co-transfection experiments, Maruyama et al. observed a dominant negative effect of rER- β 2 on estrogen activation of an ERE reporter gene by rER- β 1, whereas Petersen et al. concluded that the relative expression of the two receptors modulates the effective dose of E2 required to obtain the maximal response. The discrepancies observed between these two studies may result from the use of different rER- β 2 protein preparations (i.e fusion protein expressed in E.coli versus protein extract of rER- β 2 transfected into 293T cells, for Maruyama's and Petersen's studies, respectively), and/or the amino-acid differences mentioned earlier in the text. Differences in transactivation studies may also be

attributed to the different cell systems used, Cos cells and 293S cells for Maruyama's and Petersen's study, respectively. The deletion of the amino-acids encoded by the exon 3, which causes the deletion of the second zinc finger, results in rER- β 1 Δ 3 and rER- β 2 Δ 3 both being unable to bind a consensus ERE [10]. The affinity for E2 of rER- β 1 Δ 3 and rER- β 2 Δ 3 does not differ from that observed for rER- β 1 and rER- β 2, respectively [10].

Mouse

As for rER- β 1, mER- β 1 has been shown to bind E2 with a K_d value of 0.5 nM [14]. It has also been established that mER- β 1 is able to bind in vitro to a consensus ERE sequence [14, 16]. Ligands such E2, 4OHTam or ICI182,780 do not have any effect on mER- β 1/ERE binding, even though different experimental conditions (i.e changing the pre-incubation temperatures) may highlight a role of E2 on the formation of mER- β 1/DNA complex [14]. Co-transfection experiments performed using an ERE-driven reporter gene and mER- β 1 expression constructs into Cos and Hela cells demonstrated the activation of mER- β 1 by E2 (10 nM). This activation activity was antagonized by 4-OH-Tam and ICI182,780 [14, 16]. The co-transfection of the steroid receptor co-activator SRC-1, previously shown to interact with and to activate hER- α [26, 27], increased both ligand-dependent and -independent mER- β 1 activation [14]. Tremblay et al. showed that 4-OH-Tam and ICI182,780 were able to interfere with SRC-1 mediated ligand independent activation of mER- β 1 [14]. Similar to previous studies with ER- α [28, 29], Tremblay et al. also demonstrated that the activation of mER- β 1 could be increased via activation of the Ras-Raf-1-MAPK kinase-MAPK pathway [14]. The complete disappearance of this potentiation in the presence of ICI182,780 but not of 4-OH-Tam led the authors to conclude that the Ras mediated effects are likely mediated by a putative AF-1 domain located within the N-terminal region of mER- β 1. The target of MAPK phosphorylation was identified as the Serine residue located at position 60 of mER- β 1 sequence [14]. mER- β 1, like rER- β 1, can form homo-dimers or hetero-dimers with mouse and human ER- α [16]. mER- β 2, like rER- β 2, is able to bind to an ERE consensus sequence in vitro, does not bind E2 in ligand-binding assays but inhibits the trans-activation of mER- β 1 on classical ERE-tk-CAT reporter genes (Lu, B. personal communication). No data have been published so far regarding the putative functions of mER- β 1med, mER- β 1long, mER- β 1 Δ 5, mER- β 1 Δ 6 or mER- β 1 Δ 5-6.

Human

Similar to hER- α , hER- β 1short binds consensus ERE sequences in vitro [30]. Using various experimental conditions (different pre-incubation temperatures), Pace et al. showed that whereas ER- α and hER- β 1short bind DNA in a similar manner in the presence of E2 or tamoxifen, their DNA-binding capabilities were slightly different in the absence of ligand or in the presence of the anti-estrogen ICI182,780 [30]. Transfection experiments performed using different cell systems (CHO, Cos 7 or Hela cells), revealed that, as observed in rodents, hER- β 1short also activates the transcription of an ERE-driven reporter gene in the presence of E2 [17, 31, 32]. Moreover, as previously demonstrated for hER- α , anti-estrogens such tamoxifen, raloxifene, EM-800 or ICI164,384 are able to suppress the estrogen activation of hER- β 1short [17, 32, 33]. Similar to the rER- β 1 and in contrast to hER- α , hER- β 1short activates the transcription of AP1-regulated genes when bound to anti-estrogens such raloxifene, ICI164,384 or tamoxifen [25]. These effects were observed in several different cell types such as human endometrial carcinoma Ishikawa cells or human epithelial breast cancer MCF-7 cells. Barkhem et al. also showed that ER- α and hER- β 1short respond differently to some other synthetic estrogen agonists or antagonists [31]. For example, 17 α -ethynyl,17 β -estradiol selectively potentiates ER- α whereas 16 β ,17 α -epiestriol has selective agonist properties via hER- β 1short. Similarly, while agonistic effects of tamoxifen and raloxifene are observed in several cell systems (Cos 7, Saos, HG63 and Hela cells) using ERE-CAT reporter constructions via hER- α , no effect can be observed using hER- β 1short [32].

Taken together, all of these observations suggest that differences in estrogen and anti-estrogen action can occur when the ligands are bound to hER- α or hER- β 1, which are promoter and possibly cell type specific. Like rodent ER- β proteins, hER- β 1short can form homo-dimers as well as hetero-dimerize with hER- α [30, 34]. Hetero-dimerization of the two receptors occurs independently of ligand through the DNA-binding domain of hER- α [30]. Recently, Ogawa et al. demonstrated that ERbetacx, also known as hER- β 2, was not able to bind DNA. However, this is contrast to the observation of Moore et al. [20]. Erbetacx was unable to bind E2 and in a transient transfection experiment did not transactivate an ERE-reporter gene [21]. Ogawa et al. also showed that hER- β 2 was able to form hetero-dimers with hER- α as well as with hER- β 1long, although a preference for hetero-dimerization with hER- α was noted. Interestingly, hER- β 2 can act as a dominant negative inhibitor of hER- α induced but not hER- β 1long

induced transcription. All possible combinations of homo- and hetero-dimers have been observed between hER- β 1long, hER- β 2, hER- β 3 and hER- α [19, 20]. No function has yet been attributed to the truncated variants hER- β 3, hER- β 4, hER- β 5, hER- β 1 Δ 5, hER- β 1 Δ 6 or hER- β 1 Δ 5-6. As described previously for hER- α by Ince et al. [35], Ogawa et al. demonstrated that a C-terminally truncated hER- β 1long construct could act as a dominant negative inhibitor of hER- α and hERb1long transactivation of an ERE-driven reporter gene [36]. Similarly, a naturally occurring truncated variant of hER- α , encoded by an exon-7 deleted variant mRNA, has been shown to act as a dominant negative inhibitor of wild-type hER- α action [37]. In the light of such data plus those accumulating regarding hER- β 2 [21], it could be suggested that hER- β 3, hER- β 4 and hER- β 5 (truncated in the amino acids encoded by exon 8 of the wild-type hER- β 1 cDNA) may share similar inhibitory functions on hER- α transcriptional activity. As for hER- β 1 Δ 5, one could speculate, by analogy to what has been described for the variant encoded by an exon-5-deleted ER- α mRNA, that this isoform may have constitutive transcriptional activity [38].

TISSUE EXPRESSION

The possible expression of all ER- β isoforms mentioned above limits the interpretation of ER- β tissue expression studies published to date. In situ detection of ER- β protein or mRNA is performed using specific targeted probes that cover only limited regions of the molecule and would fail to give any information regarding what exact isoform is detected. For example, antibodies raised against the N-terminal region of hER- β 1short may allow the detection of hER- β 1long, hER- β 1short, hER- β 2, hER- β 3, hER- β 4, hER- β 5, hER- β 1 Δ 5, hER- β 1 Δ 6 and hER- β 1 Δ 5-6 isoforms. Inversely, an antibody raised against the C-terminal extremity of hER- β 1short molecule may also recognize hER- β 1long and hER- β 1 Δ 5-6 and may or may not recognize hER- β 2, hER- β 3, hER- β 4 or hER- β 5 depending on the exact localization of the epitopes recognized. Moreover, we cannot exclude the existence of other still unknown isoforms that may also be recognized by such antibodies. The difficulty of interpretation of immunocytochemical results obtained using C-terminal and N-terminal antibodies able to differentially recognize several truncated variants was previously highlighted for hER- α [39] and has recently being reported by Rosenfeld et al. for mER- β 1 immuno-detection [40]. Similar limitations exist regarding the in situ detection of ER- β related mRNAs. The previous demonstration of different sized ER- β like transcripts observed in testis or in ovary by Northern

blot analysis [11, 14] supports the expression of several different ER- β related mRNAs, an observation which should not be neglected when interpreting in situ hybridization results. Similarly, results obtained using other techniques such as Western blot, RT-PCR, RNase protection assay, or Northern blot, performed on homogenized tissue extracts, not only fail to give any information regarding the cell-specific pattern of expression of the molecules detected, but also are limited due to the heterogeneity of the detectable molecules. For example, hER- β 2 is expected to migrate on a SDS-PAGE gel with an apparent mass of 55.4 kDa and cannot be discriminated from hER- β 1short (54.2 kDa) by Western blot analysis. Similarly, even though the detection of variant isoforms by RT-PCR, performed using primers recognizing a specific variant, will provide the proof of the existence of a molecule with this particular sequence, no information about other regions of this molecule will be obtained. For example detecting ER- β 4 using a primer in exon 7 and another primer specific for hER- β 4 3' sequences will not answer the question: Does this isoform correspond to hER- β 1short, hER- β 1long or any other variant such as hER- β 1 Δ 5-6? Table 4, Table 5 and Table 6 summarize the data reporting the detection of ER- β related molecules in rat, mouse and human tissues, respectively. These tables should be read keeping in mind that the method of detection of ER- β 1 might also include several other isoforms, and that in none of these studies have probes been used which would establish whether the short, the medium or the long forms of ER- β 1 were detected. Moreover the detection of other isoforms refers only to the detection of a region of the molecule recognized by the probe used. The particular mode of detection, i.e RT-PCR, in situ hybridization, Northern blot and RNase protection assay or Western blot and immunohistochemistry is indicated. The sensitivity of the technique used to detect ER- β related molecules together with the probe used might indeed be responsible for some discrepancies between studies. The ER- β gene is apparently expressed in a significant number of tissues. Some of these tissues, like breast, uterus or ovary, are known to depend on estrogens for their growth and their differentiation. On the other hand, some of the tissues expressing ER- β related molecules, such as spleen, lung or kidney are not usually considered as "conventional" targets for estrogen action. The level of expression of a particular ER- β isoform may vary significantly from one tissue to another [6, 10, 23, 53]. For example, studies performed on rodent tissues using in situ hybridization and RNase protection assay reveal a much higher expression of rER- β 1 and mER- β 1 mRNA in the prostate and ovary than in the epididymis, the testis or the uterus [6, 23]. Similarly, PCR analysis of hER- β 4 expression revealed a strong signal in human testis but a weak one in spleen or

mammary gland [20]. For a given tissue, the level of expression of a particular isoform may also differ from one species to another. For example, the expression of ER- β 1 is high in rat prostate, but apparently low in human prostate [11, 53]. Moreover, the expression of some isoforms may also be species specific: no equivalent of the inserted variant rER- β 2 or mER- β 2, strongly expressed in multiple rodent tissues, can be detected in the corresponding human tissues [15]. This last observation, which suggests that different mechanisms of estrogen signal transduction may exist between rodent and human suggests caution in the interpretation of data where rodent models have been used to study human estrogen dependent diseases, such as breast cancer. Within a single tissue, the cells expressing ER- α and ER- β may differ. For example Prins et al. showed that rat cells expressing rER- β 1 were the prostatic epithelial cells whereas rER- α is expressed within the prostatic stroma [42, 60]. Similarly, hER- β is detected in developing spermatids but not in Leydig cells of human testis, whereas hER- α is not expressed in spermatids but is expressed in Leydig cells in human testis [11, 61].

To date, the biological significance of the expression of multiple ER- β isoforms remains to be established for each tissue. Amongst other effects, estrogen was shown to prevent bone loss resulting from osteoporosis [62], to exert a protective effect against atherosclerosis [63], to prevent the neuronal loss associated with Alzheimer's disease [64, 65] and to increase the risk of breast tumorigenesis [66, 67]. The detection of ER- β gene expression in bone, vascular system, brain and mammary gland therefore suggests a possible role of ER- β isoforms in estrogen signal transduction in these tissues. Implications of such expression have been discussed in several articles [52, 68- 73]. The rest of this article will focus on the possible role of ER- β expression in human breast cancer.

hER- β AND BREAST CANCER

Estrogens, that regulate the normal growth of human mammary tissue, are also involved in the progression of mammary cancer [66]. Before the discovery of ER- β , the effects of estrogens were thought to be mainly mediated through ER- α . The presence of variant ER- α proteins, that would be encoded by the numerous ER- α variant mRNAs detected in breast tissue, has led to the hypothesis that they could interfere with wild-type hER- α signaling pathways and therefore contribute to the apparent loss of estrogen sensitivity observed

during breast tumorigenesis [74]. It has been suggested that estrogen responsiveness may be influenced by a certain balance between all ER- α isoforms. A change in this balance, resulting in a change in estrogen responsiveness and sensitivity, may underly breast tumorigenesis and breast tumor progression [75]. Indeed, several groups have reported changes in the relative expression of particular hER- α variants during breast tumorigenesis. For example hER- $\alpha\Delta 5$, hER- $\alpha\Delta 7$ and clone 4 mRNAs (a truncated hER- α variant mRNA) were found more highly expressed in tumor tissue than in normal breast tissue [76, 77]. Similarly a higher expression of clone 4 correlated with parameters of poor prognosis and endocrine insensitivity [78]. A higher expression of hER- $\alpha\Delta 5$ was also detected in ER-/PR+ than in ER+/PR+ tumors [38], whereas ER+/PR+ tumors expressed a lower level of hER- $\alpha\Delta 7$ than ER+/PR- tumors [37]. These data support the observation that a change in hER- α isoforms balance occurs during breast tumor progression. Several studies have now described the expression of ER- β related mRNAs in normal and neoplastic human breast tissues (see Table 4, and references [11, 15, 18, 58, 79]). The functional features of some of these ER- β isoforms, underlined above in the text, suggest that ER- β proteins not only participate in the mediation of E2 effects in breast but may also modulate hER- α signal transduction. Such modulation may result from the competition for ligand, for DNA-binding, for co-activators and/or via numerous possible hetero-dimerization combinations. One could hypothesize that the number of possible combinations may also be increased by yet to be demonstrated ER- α variants/ER- β variants hetero-dimerization. It is reasonable to assume that estrogen action in breast tissue will depend therefore on the balance between all hER- α and hER- β isoforms. We first reported in 1997 the presence of hER- $\beta\Delta 5-6$ variants in breast tissue and suggested that hER- β variants may also be involved in the mechanisms underlying tumor progression [22]. The increasing number of hER- β isoforms identified to date stresses the need to determine expression levels and possible changes in hER- α /hER- β isoforms in both normal and neoplastic human breast tissue. Using a semi-quantitative RT-PCR approach to investigate the relative proportion of ER- α and ER- β in matched human normal and tumor breast samples, we have recently reported that changes occur in the balance between the two receptor [79]. We showed that in a cohort of ER+ breast tumors, a higher ER- α /ER- β ratio was observed in the tumor compared with that of the matched adjacent normal tissue component. The increased ER- α /ER- β ratio resulted primarily from an increase in ER- α mRNA expression in conjunction with in most cases, a decrease of ER- β mRNA levels in the tumor compared to the normal tissue. These data therefore suggest that the balance between the two

receptors is modified during breast tumorigenesis. Interestingly, a change in ER- α and ER- β signaling pathways, as determined by the relative expression of ER- α and ER- β , occurs during ovarian tumorigenesis [56]. Brandenberger et al. demonstrated that the expression of ER- α was equal or higher in ovarian cancer than in normal ovary whereas in contrast, ER- β expression was lowered in tumor tissue. A change in ER- β expression has also been described in chemically transformed human breast epithelial cells [80]. In contrast to the two studies mentioned above, these authors described an increase in ER- β mRNA levels paralleling transformation. One should note that even though the ER- β change does not go in the same direction, i.e. decreasing from normal to neoplastic, the modification of expression also describes a shift in the balance between the two receptors.

CONCLUSION

Since its cloning in 1995, ER- β has been the object of an extensive research effort. The increasing knowledge of the functional features of each ER- β isoform, together with its relative expression compared to other ER isoforms will hopefully soon allow a better understanding of its mechanism of action, alone or in the presence of other ER- α or ER- β molecules. However, the system is obviously complex, with many possible players as outlined in this review. Important questions still remain to be addressed: How many isoforms still remain to be identified? Do specific EREs exist for the different homo- and hetero-dimer combinations? What technique could be used to identify with certainty the isoforms observed? What are the genes specifically regulated by the the different homo- and hetero-dimer combinations? What are the actions of the different ligands on the different hetero-dimers? Do differences in the relative expression of various ER- β or ER- α variant isoforms underly well known species differences with respect to antiestrogen action, and/or the development of antiestrogen resistance in human breast cancer?

Together with other exciting avenues of research with respect to estrogen action, i.e. specific ER mediators (SERM) and coactivators/corepressors [81, 82], the existence of two ERs (ER- α or ER- β) and their multiple variant isoforms provides an incredibly exciting and challenging research environment, the results of which will impact significantly in many areas of human health.

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TABLE LEGENDS

Table 1

Functional features of rat, mouse and human ER- β isoforms. For each isoform, the possibility to bind E2 (+ or -), to recognize an ERE in a gel shift assay (+ or -), or to activate an ERE-reporter gene (+ or -) in co-transfection assays is indicated. + - refers to studies with discrepant results. The effects of estrogens or antiestrogens on AP1-driven reporter genes are also indicated. The molecules shown to form hetero-dimers with each isoform are cited. Ref: references.

Table 2

Tissue detection of rat ER- β isoforms. For each tissue, the isoform detected as well as the mode of detection. ER- β mRNAs were detected using RNase protection assay (R), in situ hybridization (I), Northern blot (N) or RT-PCR (P). ER- β proteins were detected using immuno-histochemistry (H) or Western blot (W).

Table 3

Tissue detection of mouse ER- β isoforms. For each tissue, the isoform detected as well as the mode of detection. ER- β mRNAs were detected using RNase protection assay (R), in situ hybridization (I), Northern blot (N) or RT-PCR (P). ER- β proteins were detected using immuno-histochemistry (H) or Western blot (W).

Table 4

Tissue detection of human ER- β isoforms. For each tissue, the isoform detected as well as the mode of detection. ER- β mRNAs were detected using RNase protection assay (R), in situ hybridization (I), Northern blot (N) or RT-PCR (P). ER- β proteins were detected using immuno-histochemistry (H) or Western blot (W). NB: two studies were performed on monkey tissues [57, 59]

FIGURE LEGENDS

Figure 1

Structural and functional domains of rat estrogen receptor alpha (rER- α) and beta (rER- β). Region A/B of the receptor is implicated in trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is involved in ligand-binding and another trans-activating function (AF-2). For each receptor, the length (aa), the calculated mass (kDa) and the amino acid positions of the different domains are given. Percentage amino acid identity in each domain is indicated.

Figure 2

Rat ER- β isoforms. All ER- β isoforms are aligned. Amino acid positions of the different structural domains are indicated for rER- β 1 (Genbank RNU57439, AF042058). White boxes indicate identity of amino acid between sequences. Discrepancies in all published amino acid sequences at position 27, 105, 120, and 460 of rER- β 1 are indicated by asterisks. The eighteen amino acid insertion within the LBD/AF2 domain observed in rER- β 2 ([8], Genbank RNAJ2603, AF42059, AB012721) is indicated by a gray box. rER- β 2 Δ 3 (Genbank AF42061) and rER- β 1 Δ 3 (Genbank AF42060) are missing the second zinc finger of the DNA-binding domain encoded by exon 3. rER- β 1long (Genbank RNAJ2602) contains 64 additional N-terminal amino acids. For each receptor, the length (aa) and the calculated mass (kDa) are given.

Figure 3

Mouse ER- β isoforms. All ER- β isoforms are aligned. Amino acid positions of the different structural domains are indicated for mER- β 1 (Genbank MMU81451, MMAJ220). White boxes indicate identity of amino acid between sequences. Discrepancies in all published amino acid sequences at positions 2, 97, 155, 333, 367, 400 and 466 of mER- β 1 are indicated by asterisks. The eighteen amino acid insertion observed in mER- β 2 [15] is depicted by a gray box. mER- β 1 Δ 5 and mER- β 1 Δ 6 [15] are truncated and contain different C-terminal amino acids (black boxes). mER- β 1 Δ 5-6 is missing 91 amino acid within the LBD/AF2 domain [15]. mER- β 1med (Genbank AF063853) and mER- β 1long (Genbank AF067422) contain 45 and 64 additional N-terminal amino acids, respectively. For each receptor, the length (aa) and the calculated mass

(kDa) when known or corresponding to the short (S), the medium (M) or the long (L) forms of the putative proteins are given. Broken boxes and question marks indicate that flanking amino acid sequences are unknown.

Figure 4

Human ER- β isoforms. All ER- β isoforms are aligned. White boxes indicate identity of amino acid between sequences. Amino acid positions of the different structural domains are indicated for hER- β 1short [11] that contains 8 extra N-terminal amino acids compared to the first hER- β described [17]. hER- β 1long (AF051427) contains 45 additional N-terminal amino acids. hER- β Δ 5 [15, 18], hER- β Δ 6 [15], hER- β 2 (Genbank AF051428, AB006589cx), hER- β 3 (Genbank AF060555), hER- β 4 (Genbank AF061054), hER- β 5 (Genbank AF061055) are truncated and contain different C-terminal amino acids (black boxes). hER- β Δ 5-6 ([15], Genbank AF074599) is missing 91 amino acids within the LBD/AF2 domain. For each receptor, the length (aa) and the calculated mass (kDa) when known or corresponding to the short (S) or the long (L) forms of the putative proteins are given. Broken boxes and question marks indicate that flanking amino acid sequences are unknown.

Figure 5

Exonic structure of human ER- α and ER- β . The exon structure of hER- α , hER- β , mER- β and rER- β cDNA are schematically depicted. The nucleotides are numbered starting at 1 for the ATG corresponding to the first methionine codon of the longest ER- β 1 transcript observed for each species, i.e hER- β 1long (Genbank AF051427), mER- β 1long (Genbank AF067422) and rER- β 1long (Genbank RNAJ2602).



1 190 256 360 554 600 aa, 67.0 kDa

--	--	--	--	--

1 104 170 259 455 485 aa, 54.2 kDa

--	--	--	--	--

17% 95% 29% 60% 17%



	1	104	170	259	455	
		*	*		*	
rER-β1						485 aa, 54.2 kDa
rER-β2						503 aa, 56.2 kDa
rER-β2Δ3						464 aa, 51.2 kDa
rER-β1Δ3						446 aa, 49.7 kDa
rER-β1long						549 aa, 61.3 kDa

18 aa

18 aa



1 104 170 259 455

*	*	*	*	*	*	*
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485 aa, 54.0 kDa

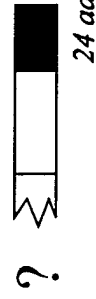
mER- β 1



?

503 aa S, 56.1 kDa
548 aa M, 61.0 kDa
567 aa L, 63.0 kDa

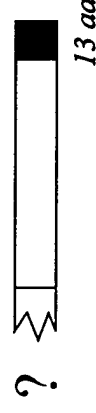
mER- β 2



?

297 aa S, 33.1 kDa
342 aa M, 38.0 kDa
361 aa L, 40.2 kDa

mER- β 1 Δ 5



?

332 aa S, 37.1 kDa
377 aa M, 42.1 kDa
396 aa L, 44.2 kDa

mER- β 1 Δ 6



?

394 aa S, 43.6 kDa
439 aa M, 48.5 kDa
458 aa L, 50.6 kDa

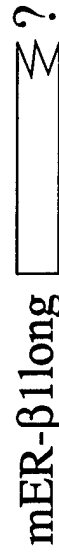
mER- β 1 Δ 5-6



?

530 aa, 59.0 kDa

mER- β 1med



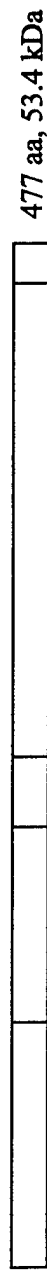
?

549 aa, 61.1 kDa

mER- β 1long



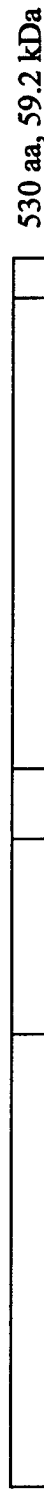
hER-β



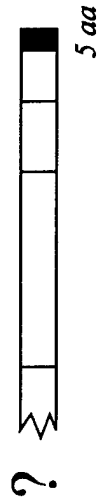
hER-β1short



hER-β1long



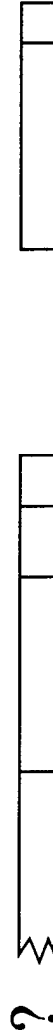
hER-β Δ5



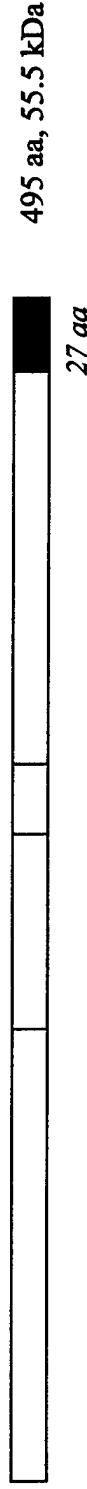
hER-β Δ6



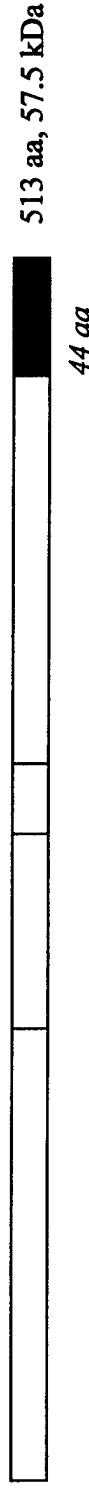
hER-β Δ5-6



hER-β2



hER-β3

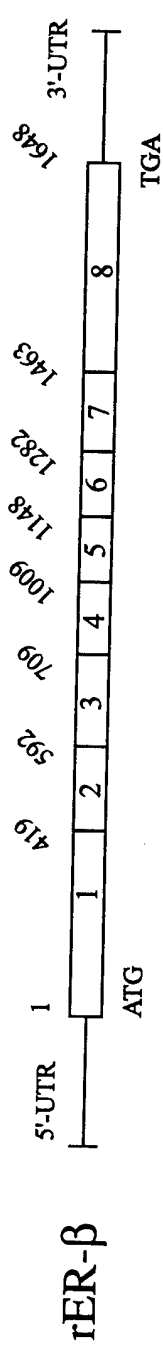
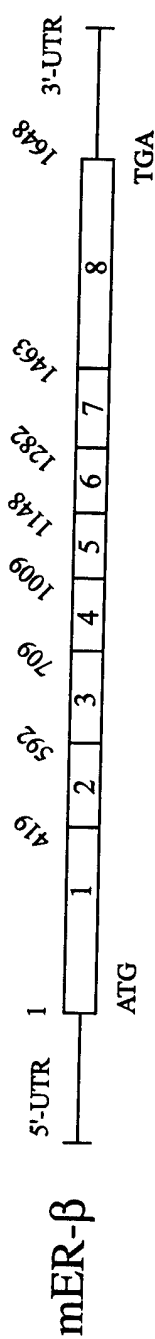
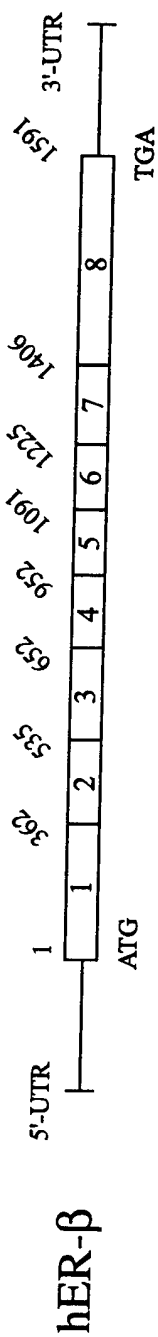
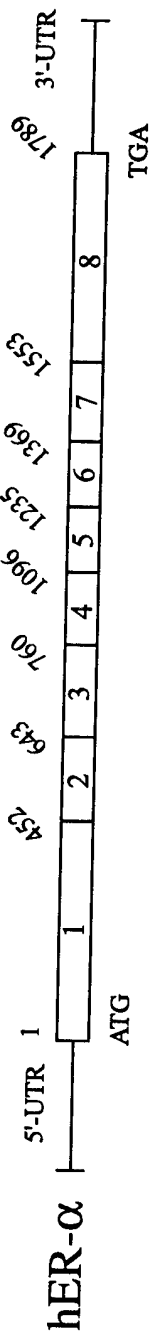
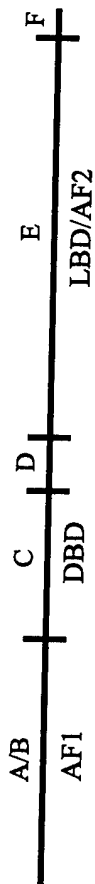


hER-β4



hER-β5





	E2 binding	ERE binding	ERE activation	Activation AP1-reporter	Hetero- dimers	Ref
rER-β1	+	+	+	- Estrogens + Antiestrogens	rER- β 1 rER- α rER- β 2	6, 9, 10, 25
rER-β2	+ -	+	+ -		rER- β 1 rER- α rER- β 2	9, 10
rER-β2Δ3	+ -	-				10
rER-β1Δ3	+	-				10
mER-β1	+	+	+		hER- α mER- α	14, 16
hER-β1short	+	+	+	- Estrogens + Antiestrogens	hER- β 2 hER- α hER- β 3	17, 25, 30, 31, 32, 33
hER-β1long	+	+	+		hER- α	19, 20
hER-β2	-	+ -	-		hER- β 1 hER- α hER- β 3	20, 21
hER-β3		+			hER- β 1 hER- α hER- β 2	20

	rER-β1	rER-β2	rER-β1Δ3	rER-β2Δ3
Prostate	P: 43,42,44,8,10, 23, 9 I: 42,6 H: 41 R: 10	P: 8,10,9 R: 10	P: 10	P: 10
Testis	P: 44,23 H: 41,45			
Peritubular cells	H: 45			
Sertoli cells	H: 45			
Leydig cells	H: 45			
Epididymis	P: 8	P: 8		
Ovary	P: 46,8,10,23,9 N: 46 R:10	P: 8,10,9 R: 10	P: 10	P: 10
Granulosa cells	I: 46,6 H: 41			
Corpus luteum	I: 47,46 H: 41			
Oviduct	H: 41			
Uterus	P: 44,8,10,23,9 N: 48 H: 41	P: 8,10,9 R: 10		
Brain	P: 9,10 R: 10			
Pituitary	P: 8,10	P: 8,10		
Supraoptic nucleus	H: 49 I: 49,50,51			
Paraventricular nucleus	H: 49 I: 49,50,51			
Hippocampus	P:10			
Hypothalamus	P: 10 I: 51	P: 10	P: 10	P: 10
Liver	P:9			
Muscle	P: 10	P: 10		
Duodenum	P: 48			
Antral Mucosa	P: 48			
Fundic Mucosa	P: 48 N: 48			
Bladder	H: 41 P: 23			
Adrenal	H: 41			
Kidney	P: 9			
Lung	H: 41 P: 23,9			
Thymus	P: 44,8 H: 41	P: 8		
Spleen	P: 44			
Bone	P: 44,9			
Heart	W: 52			
Aorta	P: 10		P: 10	P: 10

	hER-β1	hER-β2	hER-β4	hER-β5	hER-βΔ5	hER-βΔ5-6	hER-βΔ6
Prostate	P: 20	P: 20, 21		P: 20			
Testis	P: 20,55 N: 11,17	P: 20, 21	P: 20	P: 20			
Elongated spermatids	I: 11						
Ovary	P: 20,55,57,15 N: 11,17,56 I: 11	P: 20,21	P: 20	P: 20	P: 15	P: 15	P: 15
Granulosa cells	I: 11						
Uterus	P: 20,11,55,57,15	P: 20	P: 20	P: 20	P: 15	P: 15	P: 15
Breast	P: 20,11,15 I: 11		P: 20	P: 20	P: 15	P: 15	P: 15
Breast Tumor	P: 11,15,18, 58, 22 I: 11				P: 15,18	P: 22, 15	P: 15
Brain	P: 20,55						
Hippocampus	P: 59						
Hypothalamus	P: 59						
Liver	P: 20			P: 20			
Muscle	P: 20, 55	P: 20		P: 20			
Colon	P: 20,55 I: 11	P: 20		P: 20			
Duodenum	I: 11						
Small intestine		P: 20		P: 20			
Fat	P: 20	P: 20		P: 20			
Kidney	I: 11 P: 55			P: 20			
Adrenal cortex	I: 11 P: 55						
Lung	I: 11 P: 55			P: 20			
Thymus	P: 20,55 N: 17	P: 20,21	P: 20	P: 20			
Spleen	P: 20,55	P: 20	P: 20	P: 20			
Bone marrow	P: 20			P: 20			
Aorta	P: 55,57						
Heart	P: 20,55			P: 20			

	mER-β1	mER-β2	mER-β1Δ5	mER-β1Δ5-6	mER-β1Δ6
Prostate	R: 53				
Testis	P: 40				
Elongated spermatids	H: 40				
Leydig cells	H: 40 P: 40				
Epididymis	R: 53 P: 40				
Ovary	R: 53 N: 14 P: 15	P: 15	P: 15	P: 15	P: 15
Uterus	P: 15	P: 15	P: 15	P: 15	P: 15
Mammary gland	P: 15	P: 15	P: 15	P: 15	P: 15
Hippocampus	I: 54				
Hypothalamus	R: 53 I: 54				
Paraventricular nucleus	I: 54				
Liver	R: 53				
Bladder	H: 40				
Lung	R: 53				
Heart	R: 53				

APPENDIX 3

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Altered expression of estrogen receptor alpha variant mRNAs between adjacent normal breast and breast tumor tissues.

Submitted, Breast Cancer Res.

ALTERED EXPRESSION OF ESTROGEN RECEPTOR ALPHA VARIANT mRNAs BETWEEN ADJACENT NORMAL BREAST AND BREAST TUMOR TISSUES*

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Running title

ER variants in normal and tumor breast tissues

Key words

Breast cancer, Estrogen receptor, tumorigenesis, variant mRNAs.

Abbreviations

ER, estrogen receptor; PR, progesterone receptor; ERC4, ER variant truncated after sequences encoding exon 2 of the wild-type ER alpha mRNA; ERD5, exon 5 deleted ER variant; ERD3, exon 3 deleted ER variant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Statement of findings

Several variant forms of the estrogen receptor alpha (ER alpha) mRNA have been described in human breast tissues. Among these, ER alpha mRNA variants truncated after sequences encoding exon 2 of the wild-type ER alpha mRNA (ERC4 mRNA), or deleted in exon 3 (ERD3 mRNA) or exon 5 (ERD5 mRNA) sequences, were previously shown to be differentially expressed between independent normal and breast tumor tissues. Using semi-quantitative reverse transcription-polymerase chain reaction assays, we have investigated the expression of these variant mRNAs relative to wild-type ER alpha mRNA in 18 samples of normal breast tissues and their adjacent matched breast tumor tissues. A general trend towards a higher ERC4 mRNA and a lower ERD3 mRNA relative expression in the tumor compartment was observed. These differences reached statistical significance when considering only the ER-positive/progesterone receptor positive ($p=0.019$) and the ER-positive ($p=0.023$) subsets, as measured by the ligand binding assay, respectively. A significantly ($p=0.035$) higher relative expression of ERD5 mRNA was observed in tumor components overall. These data demonstrate that changes in the relative expression of ER alpha variant mRNAs occur between adjacent normal and neoplastic breast tissues. We suggest that these changes might be involved in the mechanisms underlying breast tumorigenesis.

Extended abstract

Introduction

Estrogen receptors alpha and beta are thought to mediate the action of estradiol in target tissues. Several ER alpha and ER beta variant mRNAs have been identified in both normal and neoplastic human tissues. Most of these variants contain a deletion of one or more exons of the wild-type ER mRNA. The putative proteins encoded by these variant mRNAs would therefore be missing some functional domains of the wild-type receptors and might interfere with wild-type ER signaling pathways. The detection of ER alpha variants in both normal and neoplastic

human breast tissues raised the question of their possible role in breast tumorigenesis. We have previously reported an increased relative expression of exon 5 deleted ER (ERD5) mRNA and of ERC4 mRNA, another ER- α variant mRNA truncated of all sequences following the exon 2 of the wild-type ER alpha, in breast tumor samples versus independent normal breast tissues. In contrast, a decreased relative expression of exon 3 deleted ER (ERD3) mRNA in tumor tissues and cancer cell lines versus independent normal reduction mammoplasty samples has been recently reported. These data were obtained in tissues from different individuals and possible inter-individual differences cannot be excluded.

Aims

To investigate the expression of ERC4, ERD5 and ERD3 variant mRNAs in normal breast tissues and their matched adjacent primary breast tumor tissues.

Materials and methods

Eighteen human breast specimens were selected. For each case, tumor and adjacent normal tissues from the same individual were available and histologically characterized by observation of paraffin sections. Six tumors were ER-/progesterone receptor (PR)-, nine were ER+/PR+, two were ER+/PR- and one was ER-/PR+, as measured by ligand binding assay. For each specimen, total RNA was extracted from frozen normal and tumor tissue sections and reverse transcribed. The expressions of ERC4, ERD5 and ERD3 mRNAs relative to wild-type ER alpha mRNA were investigated by semiquantitative RT-PCR assays performed using three different set of primers.

Results

Relative expression of ERC4 mRNA in matched normal and breast tumor tissues

As shown Figure 1A, two PCR products were obtained, that corresponded to wild-type ER and ERC4 mRNAs. For each case, the mean of the ratios obtained in at least three independent PCR experiments is shown for both normal and tumor compartments (Figure 1B). A statistically higher ERC4 mRNA relative expression was found in the neoplastic components of ER+/PR+ tumors, as compared to matched adjacent normal tissues ($p=0.019$, Wilcoxon signed rank test).

Relative expression of ERD3 mRNA in matched normal and breast tumor tissues

Two PCR products were obtained, that corresponded to WT-ER and ERD3 mRNAs (Figure 2A). A significantly higher expression of ERD3 mRNA in the normal compared to the adjacent neoplastic components of ER+ subset (n=8, p=0.023, Wilcoxon signed rank test, Figure 2B).

Relative expression of ERD5 mRNA in matched normal and breast tumor tissues

Two PCR products were obtained, that corresponded to WT-ER and ERD5 cDNAs (Figure 3A). As shown in Figure 3B, a statistically significant higher relative expression of ERD5 mRNA was observed in tumor components when this expression was measurable in both normal and adjacent tumor tissues (n=15, p=0.035, Wilcoxon signed rank test).

Discussion

A statistically significant higher ERC4 mRNA expression was found in ER+/PR+ tumors as compared to matched normal breast tissues. ERC4 variant mRNA has been previously shown to be more highly expressed in ER positive tumors showing poor as opposed to tumors showing good prognostic characteristics. Interestingly we also have reported similar levels of expression of ERC4 mRNA in primary breast tumors and their concurrent axillary lymph node metastases. Taken together, these data suggest that the putative role of the ERC4 variant might be important in the earliest phases of breast tumorigenesis rather than in the later stages of breast cancer progression. Transient expression assays revealed that the protein encoded by ERC4 mRNA was unable to activate the transcription of an ERE-reporter gene or to modulate the wild-type ER protein activity. The biological significance of the changes observed in ERC4 mRNA expression during breast tumorigenesis remains therefore unclear.

A higher relative expression of ERD3 mRNA in the normal breast tissue components compared to adjacent neoplastic tissue was found in the ER+ subgroup. These data are in agreement with the recently published report of Erenburg et al. who showed a decreased relative expression of ERD3 mRNA in neoplastic breast tissues and breast cancer compared with independent reduction mammoplasty and breast tumor. Transfection experiments showed that the activation of the transcription of the pS2 gene by estrogen was drastically reduced in the presence of increased ERD3 expression. The authors hypothesized that the reduction of ERD3 expression could be a pre-requisite for breast carcinogenesis to proceed.

We observed a significantly higher relative expression of ERD5 mRNA in breast tumor components compared to matched adjacent normal breast tissue. These data confirm our previous observations performed on unmatched normal and neoplastic human breast tissues. Upregulated expression of this variant has already been reported in ER negative/PR positive tumors, as compared to ER positive/PR positive tumor, suggesting a possible correlation between ERD5 mRNA expression and breast tumor progression. Eventhough it has been suggested that ERD5 could be related to the acquisition of insensitivity to antiestrogen treatment (i.e Tamoxifen), accumulating data refute a general role for ERD5 in hormone-resistant tumors. Only ER positive pS2 positive tamoxifen resistant tumors have been shown to express significantly higher levels of ERD5 mRNA, as compared to control tumors. Taken together, these data suggest that the exact biological significance of ERD5 variant expression during breast tumorigenesis and breast cancer progression, if any, remains unclear.

In conclusion, we have shown that the relative expression of ERC4 and ERD5 variant mRNAs was increased in human breast tumor tissue, as compared to normal adjacent tissue, whereas the expression of ERD3 variant mRNA was decreased in breast tumor tissues. These results suggest that the expression of several ER alpha variant mRNAs is deregulated during human breast tumorigenesis. Whether or not a functional role of altered ER alpha variant expression is involved in the mechanisms underlying breast tumorigenesis remains to be determined.

Full version of the article

Introduction

Estrogen receptors alpha (ER alpha) and beta (ER beta) are thought to mediate the action of estradiol in target tissues [1, 2]. These two receptors, which belong to the steroid/retinoic acid/thyroid receptor superfamily [3], contain several structural and functional domains [4] encoded by two mRNAs containing 8 exons [5, 6]. Upon ligand binding, ER alpha and ER beta proteins recognize specific estrogen responsive elements (EREs) located in DNA in the proximity of target genes, and through interactions with several co activators modulate the transcription of these genes [7]. Several ER alpha and ER beta variant mRNAs have been identified in both normal and neoplastic human tissues [8-12]. Most of these variants contain a deletion of one or more exons of the wild-type ER mRNA. The putative proteins encoded by these variant mRNAs would therefore be missing some functional domains of the wild-type receptors and might interfere with wild-type ER signaling pathways. Indeed, *in vitro* functional studies have shown that some recombinant ER alpha variant proteins can affect estrogen

regulated gene transcription. For example, ERD3, the variant protein encoded by exon 3 deleted ER alpha mRNA, that is missing the second zinc finger of the DNA binding domain, has been shown to have a dominant negative activity on wild-type ER alpha receptor action [13]. A similar dominant negative activity has been observed for ERD5 variant protein, encoded by an ER alpha variant mRNA deleted in exon 5 sequences, that is missing a part of the hormone binding domain of the wild-type molecule [14]. Interestingly, a constitutive hormone independent activity [15] and a wild-type enhancing activity [16] have also been attributed to ERD5 variant protein in different systems. The relevance of the levels achieved in these transfection experiments to *in vivo* expression remains unclear. One should also note that these functional activities are likely to be cell type and promoter specific [8]. However, the discovery that these ER alpha variants are expressed in both normal and neoplastic human breast tissues raised the question of their possible role in breast tumorigenesis [8]. We have previously reported an increased relative expression of ERD5 mRNA and of ERC4 mRNA, another ER alpha variant mRNA truncated of all sequences following the exon 2 of the wild-type ER alpha [17], in breast tumor samples versus independent normal breast tissues [18, 19]. In contrast, Erenburg et al. reported recently a decreased relative expression of ERD3 mRNA in tumor tissues and cancer cell lines versus independent normal reduction mammoplasty samples [20]. These data, which suggested that alteration in ERD5, ERD3 and clone 4 mRNA expression might occur during breast tumorigenesis, were obtained in tissues from different individuals and possible inter-individual differences cannot be excluded.

Aims

In order to clarify this issue, we investigated the expression of these three variant mRNAs in normal breast tissues and their matched adjacent primary breast tumor tissues.

Materials and methods

Human breast tissues and reverse transcription

Human breast specimens (18 cases) were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The processing of specimens collected in the Manitoba Breast Tumor Bank has already been described [21]. Briefly, each specimen had been rapidly frozen as soon as possible after surgical removal. A portion of the frozen tissue block was processed to create a paraffin-embedded tissue block matched and orientated relative to the

remaining frozen block. These paraffin blocks provide high quality histologic sections, which are used for pathologic interpretation and assessment and are mirror images of the frozen sections used for RNA extractions. For each case, tumor and adjacent normal tissues from the same individual were histologically characterized by observation of paraffin sections. The presence of normal ducts and lobules as well as the absence of any atypical lesion were confirmed in all normal tissue specimens. Seven tumors were estrogen receptor (ER) negative (ER < 3 fmol/mg protein), with progesterone receptor values ranging from 2.2 to 11.2 fmol/mg protein, as measured by ligand binding assay. Eleven tumors were ER positive (ER values ranged from 3.5 to 159 fmol/mg protein) with progesterone receptor values ranging from 5.8 to 134 fmol/mg protein. These tumors spanned a wide range of grade (grade 5 to 9, median = 7.5), determined using the Nottingham grading system. Patient were from 39 to 86 years old (median = 54). Total RNA was extracted from frozen tissue sections and reverse transcribed in a final volume of 25 μ l as previously described [18]. The quality of cDNAs obtained was assessed by amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, as described previously [18].

Polymerase chain reaction (PCR)

ERC4 primer set was used to co-amplify wild-type ER alpha (WT-ER) and ERC4 cDNAs. This set consisted of ERU primer (5'-TGTGCAATGACTATGCTTCA-3'; sense; located in WT-ER exon 2; position 792-811), ERL primer (5'-GCTCTTCCTCCTGTTTTAT-3'; antisense; located in WT-ER exon 3; position 940-921), and C4L primer (5'-TTTCAGTCTTCAGATACCCAG-3'; antisense; located in C4ER sequence; position 1336-1315). ERD3 primer set, used to co-amplify WT-ER and ERD3 cDNAs, consisted of D3U primer (5'-TGTGCAATGACTATGCTTCA-3'; sense; located in WT-ER exon 2; position 792-811) and D3L primer (5'-TGTTCTTCTTAGAGCGTTTGA-3'; antisense; located in WT-ER exon 4; position 1145-1125). ERD5 primer set, used to co-amplify WT-ER and ERD5 cDNAs, consisted of D5U primer (5'-CAGGGGTGAAGTGGGGTCTGCTG-3'; sense; located in WT-ER exon 4; position 1060-1082) and D5L primer (5' alphaTGCGGAACCGAGATGATGTAGC-3'; antisense; located in WT-ER exon 6; position 1542-1520). The given positions correspond to WT-ER [1] and ERC4 [17] published sequences.

PCR amplifications were performed and PCR products analyzed as previously described [18, 22]. Briefly, 0.2 μ l of reverse transcription mixture was amplified in a final volume of 15 μ l, in the presence of 1.5 μ Ci of [α -³²P]

dCTP (3000Ci/mmol), 4 ng/ μ l of each primer of the primer set considered (ERC4, ERD3 or ERD5 primer set) and 0.3 unit of Taq DNA polymerase. Each PCR consisted of 30 cycles: 1 min at 94°C, 30 sec at 60°C and 1 min at 72°C for ERC4 primer set; and 30 sec 94°C, 30 sec at 60°C and 30 sec at 72°C for ERD3 and ERD5 primer sets. PCR products were then separated on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and autoradiographed. For each PCR, two PCR products were obtained, which were identified by subcloning and sequencing, performed as previously described [18]. PCR products migrating with the apparent size of 149 bp, 354 bp and 483 bp, using ERC4, ERD3 and ERD5 primer set, respectively, were shown to correspond to WT-ER cDNA. PCR products migrating with the apparent size of 536 bp, 237 bp and 344 bp, using ERC4, ERD3 and ERD5 primer set, were shown to correspond to ERC4, ERD3 and ERD5 cDNAs, respectively.

For each experiment, bands corresponding to the variant mRNA (i.e ERC4, ERD3 or ERD5) and to WT-ER, were excised from the gel and counted in a scintillation counter.

Quantitation and statistical analysis

For each set of primers (i.e ERC4, ERD3 and ERD5 primer set) and for each sample, 4 independent PCR assays were performed. The ratios between ERC4, ERD3 or ERD5 signals and corresponding WT-ER signal were calculated. For each experiment, in order to correct for overall inter-assay variations (due to different batches of radiolabelled [α -³²P] dCTP or of Taq DNA polymerase), the ratio observed in the same particular tumor (case number 12) was arbitrarily given the value of one and all other ratios expressed relatively. Under our experimental conditions, some samples did not have measurable levels (i.e signal lower than twice the background value) of D3ER or D5ER variant mRNAs (see figure 2A and 3A) in any of the 4 repetitions performed. Only cases having detectable levels in at least 3 of the replicates in both their normal and tumor compartments were included in the statistical analysis. The significance of the differences in the relative levels of expression of ERC4, ERD3 and ERD5 mRNAs between matched normal and tumor components was determined using the Wilcoxon signed rank test.

Results

In order to investigate the expression of ERC4, ERD3 and ERD5 mRNA expression relative to WT-ER mRNA within matched normal and breast tumor tissues, eighteen cases were selected in the Manitoba Breast Tumor

Bank, which had well separated and histopathologically characterized normal and adjacent neoplastic components. Total RNA was extracted from frozen tissue sections and reverse-transcribed as described in the "Materials and Methods" section. In order to assess the quality of the resulting cDNAs, PCR was performed using primers recognizing the ubiquitously expressed gene, GAPDH. Similar GAPDH signals were obtained in all samples, indicating a similar quality of all cDNA samples (data not shown).

Relative expression of ERC4 mRNA in matched normal and breast tumor tissues

A recently described triple-primer PCR assay (TP-PCR assay) was used to compare the relative expression of ERC4 mRNA between adjacent normal and tumor components [19, 22]. In this assay, three primers are used simultaneously during the PCR: the upper primer is able to recognize both WT-ER and ERC4 cDNA sequences whereas the two lower primers are specific for each cDNA. Competitive amplification of two PCR products occurs, giving a final PCR product ratio related to the initial input of target cDNAs. This approach has been validated previously both by competitive amplification of spiked cDNA preparations [19] and by comparison to RNase protection assays [22]. As shown Figure 1A, two PCR products were obtained, that migrated at the apparent size of 149 bp and 536 bp. These products have been shown to correspond to WT-ER and ERC4 mRNAs, respectively [22]. One should note the presence, in samples where WT-ER and ERC4 signals are high (see Figure 1A, lane 5), of several additional bands, one of which has been previously identified as corresponding to exon 2-duplicated ER alpha variant cDNA [22]. The presence of these minor PCR products did not interfere with the quantitative aspect of the TP-PCR assay [22]. For each case, the mean of the ratios obtained in at least three independent PCR experiments, expressed in arbitrary units, is shown for both normal and tumor compartments (Figure 1B). A trend toward a higher clone 4 mRNA relative expression in the tumor compartment was observed (12 out of 18 cases, $p=0.47$, Wilcoxon signed rank test). When considering only the ER positive/PR positive subset ($n=9$), as measured by the ligand binding assay, a statistically higher ERC4 mRNA relative expression was found in the neoplastic components, as compared to matched adjacent normal tissues ($p=0.019$, Wilcoxon signed rank test).

Relative expression of ERD3 mRNA in matched normal and breast tumor tissues

A PCR assay, performed using primers annealing to sequences in exon 2 and 4, was used to investigate ERD3 mRNA expression relative to WT-ER in these 18 matched cases. We and others have previously shown that the co-amplification of WT-ER and an exon-deleted ER alpha variant cDNA, resulted in the amplification of two PCR

products, the relative signal intensity of which provided a previously validated measurement of exon-deleted ER alpha variant expression [18, 23]. Two PCR products were obtained, that migrated with an apparent size of 354 bp and 237 bp, (Figure 2A). These fragments were shown by subcloning and sequencing to correspond to WT-ER and ERD3 mRNAs (data not shown). The relative ERD3 signal was measurable in the normal and the tumor compartments of 13 cases (see Figure 2B). Out of these 13 cases, ERD3 mRNA expression was higher in the normal compartment of 10 cases. This difference, however, did not reach statistical significance ($p=0.057$, Wilcoxon signed rank test). However, a significantly higher expression of ERD3 mRNA in the normal compared to the adjacent neoplastic components was found when only the ER positive subset was considered ($n=8$, $p=0.023$, Wilcoxon signed rank test).

Relative expression of ERD5 mRNA in matched normal and breast tumor tissues

Using primers annealing to sequences in exon 4 and 6 of WT-ER, we also investigated the relative expression of ERD5 mRNA in these 18 matched cases. Two PCR products were obtained, that migrated at an apparent size of 483 bp and 344 bp and that have previously been shown to correspond to WT-ER and ERD5 cDNAs, respectively (Figure 3A). As shown in Figure 3B, a statistically significant higher relative expression of ERD5 mRNA was observed in tumor components when this expression was measurable in both normal and adjacent tumor tissues ($n=15$, $p=0.035$, Wilcoxon signed rank test).

Discussion

The expression of ERC4, ERD3 and ERD5 variant mRNAs relative to WT-ER mRNA expression within adjacent normal and neoplastic human breast tissues was investigated using previously described semi-quantitative RT-PCR assays [18, 19, 22]. These assays allow the determination of the expression of ER alpha variant mRNA relative to WT-ER mRNA using a very small amount of starting material, and offer the advantage of allowing investigators to work with histopathologically well characterized human breast tissue regions. One should note however that the sensitivities of the assays used in this study differed from each other. The TP-PCR assay, previously set up to allow the determination of ERC4 relative expression in tumor samples with very low ER levels, as measured by ligand binding assay, gave a measurable value of expression in 36 out of the 36 samples studied. This contrasts with the detection of 30 out of 36 and 33 out of 36 obtained using ERD3 specific and

ERD5 specific primers, respectively. These differences in sensitivity probably result from different primer set efficiencies under our experimental conditions.

A trend towards a higher ERC4 mRNA relative expression in tumor components compared to the normal adjacent tissue component has been observed in the cohort studied (12 out of 18 cases). This difference reached statistical significance when considering the ER positive/PR positive subgroup ($n = 9$, $p = 0.019$). This result is in agreement with our previous data obtained by comparing ERC4 mRNA expression between independent normal reduction mammoplasty samples and a group of ER positive/PR positive breast tumors [19]. The absence of statistically significant differences when considering the total cohort in the present study may result from the low number of matched cases studied or to the different biology of ER negative cases. Further studies are needed to clarify this issue. ERC4 variant mRNA has been previously shown to be more highly expressed in ER positive tumors showing poor prognostic characteristics (presence of more than 4 axillary lymph nodes, tumor size >2 cm, aneuploid, high % S-phase cells) than in ER positive tumor with good prognostic characteristics (absence of axillary lymph node, tumor size < 2 cm, diploid, low % S-phase cells) [24]. Moreover, in this previous study, an higher ERC4 mRNA expression has also been observed in ER positive/PR negative tumors, as compared to ER positive/PR positive tumors [24]. Interestingly we also have recently reported similar levels of expression of ERC4 mRNA in primary breast tumors and their concurrent axillary lymph node metastases [22]. Taken together, these data suggest that the putative role of the ERC4 variant protein might be important in the earliest phases of breast tumorigenesis rather than in the later stages of breast cancer progression. Transient expression assays revealed that the protein encoded by ERC4 mRNA was unable to activate the transcription of an ERE-reporter gene or to modulate the wild-type ER protein activity [17]. The biological significance of the changes observed in ERC4 mRNA expression during breast tumorigenesis remains therefore unclear.

A trend towards a higher relative expression of ERD3 mRNA in the normal breast tissue components compared to adjacent neoplastic tissue was found (10 out of 13 cases), which reached statistical significance when the ER positive subgroup was only considered. These data are in agreement with the recently published report of Erenburg et al. who showed a decreased relative expression of ERD3 mRNA in neoplastic breast tissues and breast cancer compared with independent reduction mammoplasty and breast tumor [20]. Transfection experiments performed by these investigators showed that the activation of the transcription of the pS2 gene by estrogen was drastically reduced in the presence of increased ERD3 expression. Moreover, ERD3 transfected

MCF-7 cells had a reduced saturation density, exponential growth rate and *in vivo* invasiveness, as compared to control cells. These data led the authors to hypothesize that the reduction of ERD3 expression could be a pre-requisite for breast carcinogenesis to proceed. They suggested that if high levels of ERD3 could attenuate estrogenic effects in normal breast tissue, low levels might lead to an excessive and unregulated mitogenic action of estrogen.

We observed a significantly higher relative expression of ERD5 mRNA in breast tumor components compared to matched adjacent normal breast tissue. These data confirm our previous observations performed on unmatched normal and neoplastic human breast tissues. Upregulated expression of this variant has already been reported in ER negative/PR positive tumors, as compared to ER positive/PR positive tumor [15, 25], suggesting a possible correlation between ERD5 mRNA expression and breast tumor progression. Interestingly, ERD5 mRNA can be detected in human pituitary adenomas but not in normal pituitary samples [26]. This underscores the putative involvement of this ER variant in other tumor systems. Eventhough it has been suggested that ERD5 could be related to the acquisition of insensitivity to antiestrogen treatment (i.e Tamoxifen)[27, 28], accumulating data refute a general role for ERD5 in hormone-resistant tumors [14, 23, 29, 30]. Only ER positive pS2 positive tamoxifen resistant tumors have been shown to express significantly higher levels of ERD5 mRNA, as compared to control tumors [31]. Taken together, these data suggest that the exact biological significance of ERD5 variant expression during breast tumorigenesis and breast cancer progression, if any, remains unclear.

Among all the articles published so far on ER variants, only one has investigated ER variant expression between normal and neoplastic matched samples. Indeed, Okada et al. recently reported a study performed on 15 cases. They observed an apparent difference in ER variant mRNA expression between adjacent normal and tumor samples [31]. However, this study was performed using a less sensitive PCR approach, as PCR products were stained using ethidium bromide, and no attempt was made to quantify ER variant mRNA expression relative to WT-ER mRNA expression.

In conclusion, we have shown that the relative expression of ERC4 and ERD5 variant mRNAs was increased in human breast tumor tissue, as compared to normal adjacent tissue, whereas the expression of ERD3 variant mRNA was decreased in breast tumor tissues. These results, which confirm previous data obtained on independent human breast tissue samples, suggest that the expression of several ER alpha variant mRNAs is

deregulated during human breast tumorigenesis. Whether or not a functional role of altered ER alpha variant expression is involved in the mechanisms underlying breast tumorigenesis remains to be determined.

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Figures legend

Figure 1

Comparison of the relative expression of ERC4 variant mRNA between breast tumor and adjacent matched normal breast samples. A: Total RNA extracted from frozen tissue sections from tumor (T) and adjacent normal (N) breast tissue samples was reverse transcribed and PCR amplified as described in the "Materials and Methods" section using ERU, ERL and C4L primers. Radioactive PCR products were separated on a 6% acrylamide gel and visualized by autoradiography. Bands migrating at 149 bp and 536 bp were identified as corresponding to WT-ER and ERC4 variant mRNA, respectively. M: Molecular weight marker (ϕ x174 HaeIII digest, Gibco BRL, Grand Island, NY). C: negative control (no cDNA added during the PCR reaction). **B:** For each case, signals corresponding to ERC4 variant mRNA was quantified as described in "Materials and Methods" and expressed in arbitrary units for tumor (black column) and normal (white column) components. For each sample, the mean of at least three different PCR assays is indicated. Cases are sorted by ER status (black bottom lane, negative: -, or positive: +) and PR status (gray bottom lane, negative: -, or positive: +). The significance of the differences between tumor and normal matched components within each subgroup, as tested using the Wilcoxon matched pair test, is indicated when p values are lower than 0.05.

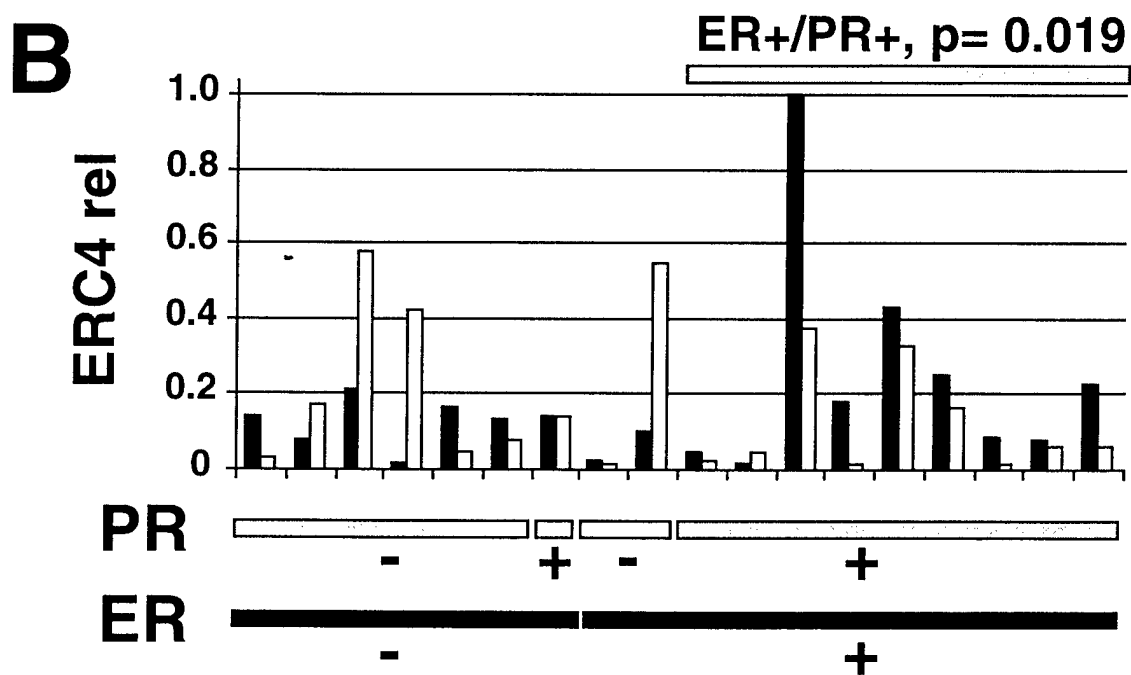
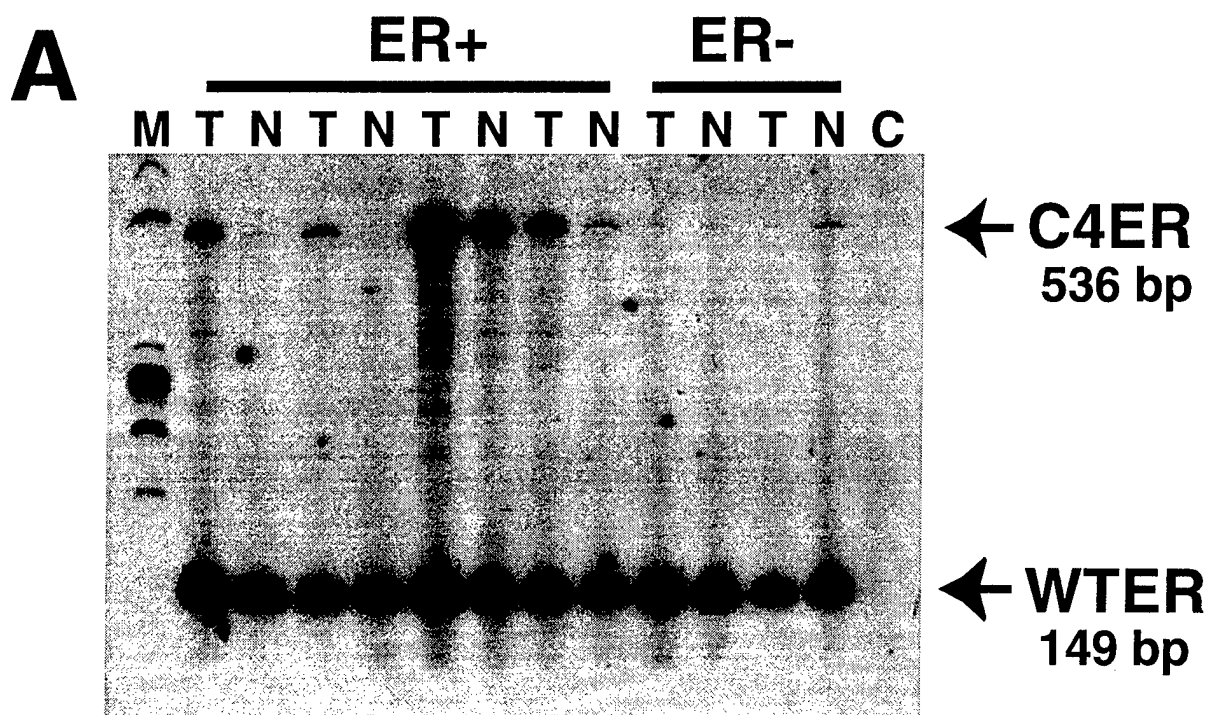
Figure 2

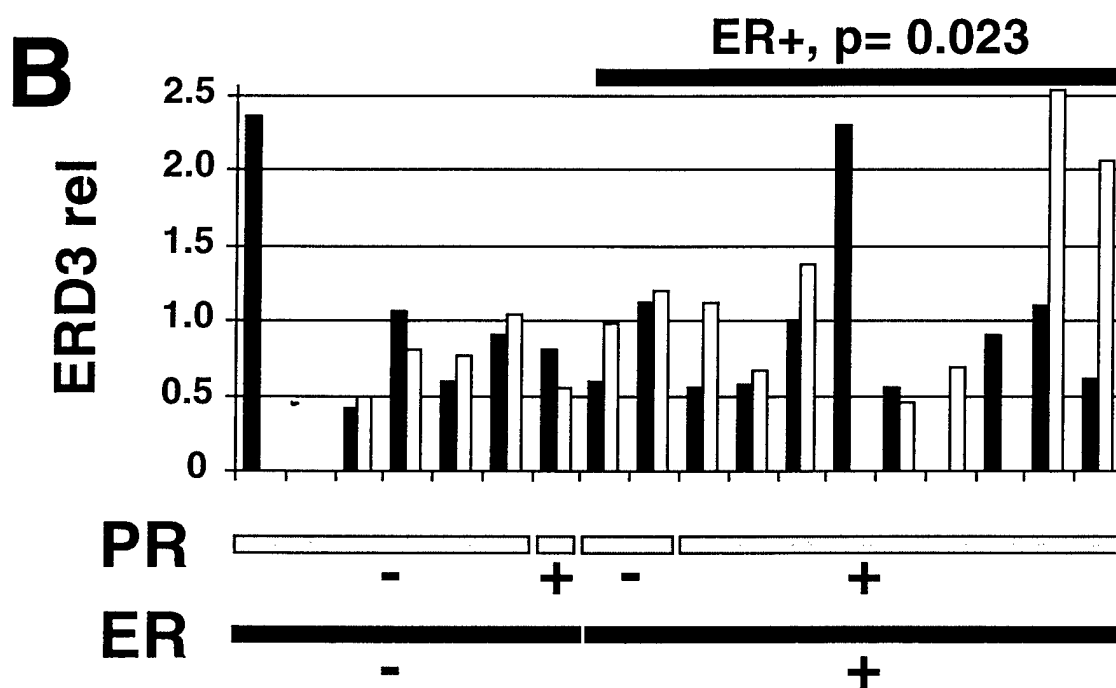
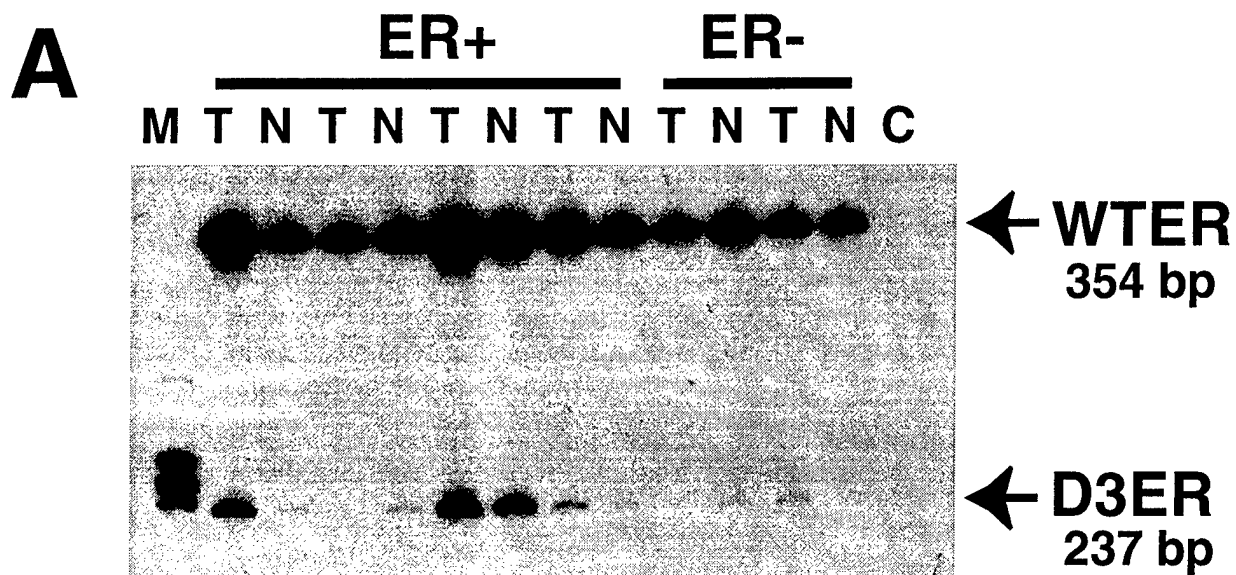
Comparison of the relative expression of ERD3 variant mRNA between breast tumor and adjacent matched normal breast samples. A: Total RNA extracted from frozen tissue sections from tumor (T) and adjacent normal (N) breast tissue samples was reverse transcribed and PCR amplified using D3U and D3L primers. Radioactive PCR products were separated on a 6% acrylamide gel and visualized by autoradiography. Bands migrating at 354 bp and 237 bp were identified as corresponding to WT-ER and ERD3 variant mRNA, respectively. M: Molecular weight marker. C: negative control. **B:** For each case, signals corresponding to ERD3

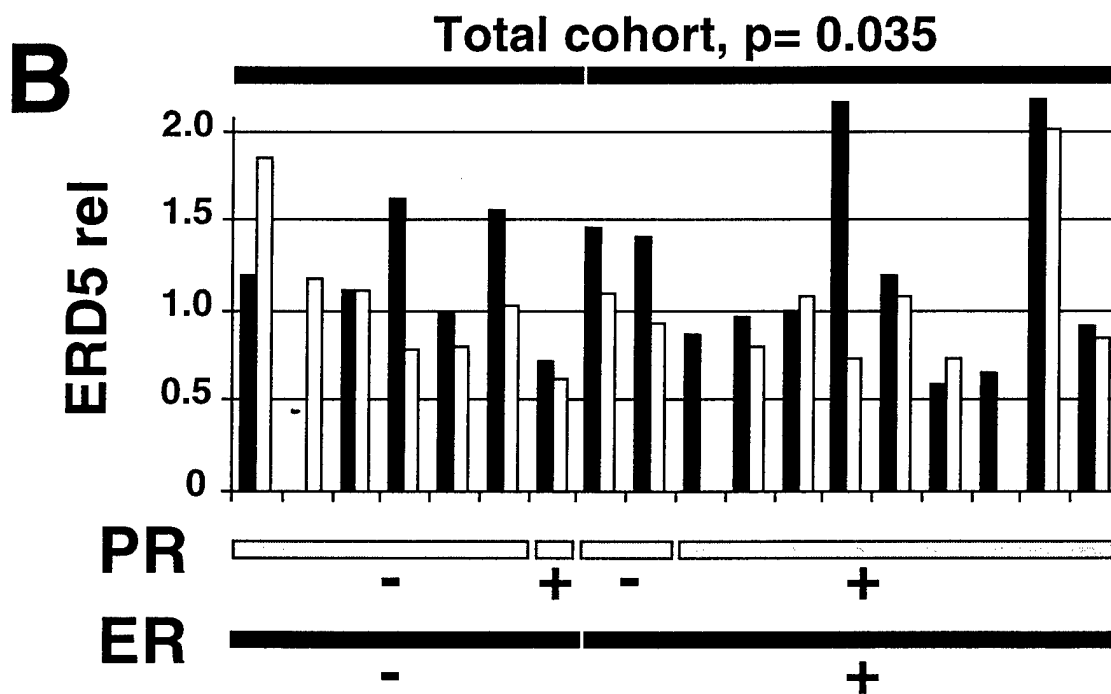
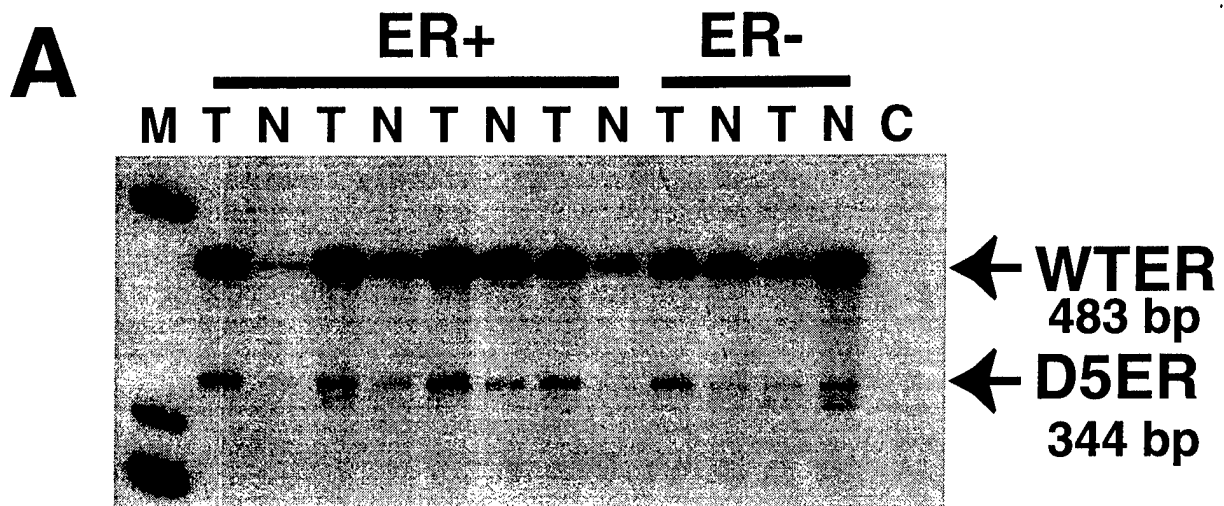
variant mRNA was quantified and expressed in arbitrary units for tumor (black column) and normal (white column) components. For each sample, the mean of at least three different PCR assays is indicated. Cases are sorted by ER status (black bottom lane, negative: -, or positive: +) and PR status (gray bottom lane, negative: -, or positive: +). Samples that failed to have three measurable signals in the four experiments performed in both normal and neoplastic components were not included in the statistical analysis. The significance of the differences between tumor and normal matched components within each subgroup, as tested using the Wilcoxon matched pair test, is indicated when p values are lower than 0.05.

Figure 3

Comparison of the relative expression of ERD3 variant mRNA between breast tumor and adjacent matched normal breast samples. A: Total RNA extracted from frozen tissue sections from tumor (T) and adjacent normal (N) breast tissue samples was reverse transcribed and PCR amplified using D5U and D5L primers. Radioactive PCR products were separated on a 6% acrylamide gel and visualized by autoradiography. Bands migrating at 483 bp and 344 bp were identified as corresponding to WT-ER and ERD5 variant mRNA, respectively. M: Molecular weight marker. C: negative control. **B:** For each case, signals corresponding to ERD5 variant mRNA was quantified and expressed in arbitrary units for tumor (black column) and normal (white column) components. For each sample, the mean of at least three different PCR assays is indicated. Cases are sorted by ER status (black bottom lane, negative: -, or positive: +) and PR status (gray bottom lane, negative: -, or positive: +). Samples that failed to have three measurable signals in the four experiments performed in both normal and neoplastic components were not included in the statistical analysis. The significance of the differences between tumor and normal matched components within each subgroup, as tested using the Wilcoxon matched pair test, is indicated when p values are lower than 0.05.







APPENDIX 4

Leygue E, Hall R, Dotzlaw H, Watson PH, and Murphy LC

Estrogen receptor- α variant mRNAs expression in primary human breast tumors and matched lymph node metastases.

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Oestrogen receptor- α variant mRNA expression in primary human breast tumours and matched lymph node metastases

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Summary We have shown previously that the relative expression of a truncated oestrogen receptor- α variant mRNA (ER clone 4) is significantly increased in axillary node-positive primary breast tumours compared with node-negative tumours. In this study, we have examined the relative expression of clone 4-truncated, exon 5-deleted and exon 7-deleted oestrogen receptor- α variant mRNAs in 15 primary breast tumour samples and in synchronous axillary lymph node metastases. Overall, there were no significant differences between the primary tumours and the matched metastases in the relative expression of these three specific variant mRNAs. Furthermore, the pattern of all deleted oestrogen receptor- α variant mRNAs appeared conserved between any primary and its matched secondary tumour.

Keywords: oestrogen receptor- α variants; breast cancer; metastasis

Multiple oestrogen receptor- α (ER) mRNA species have been identified in human breast cancer samples (Dowsett et al, 1997; Murphy et al, 1997a, b). The significance of these variant transcripts remains unclear. Although the ability to detect variant ER proteins encoded by such variant transcripts remains controversial (Park et al, 1996; Desai et al, 1997; Huang et al, 1997), alteration of expression of some variant ER mRNAs has been found to occur during both breast tumorigenesis (Leygue et al, 1996a, b) and breast cancer progression. With regard to the latter, we showed previously that the expression of the truncated, clone 4 variant (C4) ER mRNA (Dotzlaw et al, 1992) was significantly increased relative to wild-type (WT) ER mRNA in a group of primary breast tumours with multiple poor prognostic features compared with a group of primary breast tumours with good prognostic features (Murphy et al, 1995). The 'poor' prognostic features were defined as the presence of lymph node metastases at the time of surgery, large tumour size, lack of progesterone receptor (PR) expression and high S-phase fraction, while 'good' prognostic features were lack of nodal involvement, small tumour size, PR positivity and low S-phase fraction. In the same study, the relative expression of clone 4 ER variant mRNA was significantly higher in primary breast tumours that were PR- than in those that were PR+ (Murphy et al, 1995). This suggested that altered ER variant expression may be a marker of a more aggressive phenotype and lack of endocrine sensitivity in human breast cancer. As a prerequisite to addressing such a possibility, we have investigated the

pattern of ER variant expression in a cohort of primary tumours and their matched, concurrent lymph node metastases.

MATERIALS AND METHODS

Tumour selection and RNA isolation

Sections from 15 frozen primary human breast tumour samples and their matched frozen lymph node metastases were provided by the Manitoba Breast Tumour Bank (Winnipeg, MB, Canada). For the primary tumour samples, the ER levels, determined by ligand-binding assays, ranged from 0.8 fmol mg⁻¹ protein to 89 fmol mg⁻¹ protein with a median value of 17.5 fmol mg⁻¹ protein. Thirteen tumours were ER+ and two were ER- (ER+ was defined as >3 fmol mg⁻¹ protein). PR levels determined by ligand-binding assays ranged from 2.9 fmol mg⁻¹ protein to 112 fmol mg⁻¹ protein with a median value of 12.6 fmol mg⁻¹ protein. Nine tumours were PR+ and 6 were PR- (PR+ was defined as > 10 fmol mg⁻¹ protein). ER and PR values were available for only four of the lymph node metastases and the ER and PR status as defined by ligand binding did not differ from their matched primary tumour. RNA was extracted from the sections using Trizol reagent (Gibco/BRL, Ontario, Canada) according to the manufacturer's instructions.

For validation of triple-primer polymerase chain reactions (TP-PCR) by comparison with RNAase protection assays, a second cohort of human breast tumour specimens (25 cases) was also obtained from the Manitoba Breast Tumour Bank. Twenty of these tumours were ER+, as determined by ligand-binding assay, with values ranging from 4.5 to 311 fmol mg⁻¹ protein (median 93 fmol mg⁻¹). The five remaining cases were ER-, with values ranging from 0 to 1.8 fmol mg⁻¹ protein (median 0.9 fmol mg⁻¹). Total RNA was extracted from frozen tissues using guanidinium

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thiocyanate as previously described (Murphy and Dotzlaw, 1989). The integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (Murphy and Dotzlaw, 1989).

RNAase protection assay

Antisense riboprobes spanning the point at which the C4 ER mRNA sequence diverges from the WT ER mRNA sequence (Dotzlaw et al, 1992) were synthesized as previously described (Dotzlaw et al, 1990). The level of C4 ER mRNA and WT ER mRNA in 10 μ g of total RNA was determined using an RNAase Protection Assay kit (RPA II, Ambion, Austin, TX, USA) following the manufacturer's instructions. Briefly, RNA was denatured at 80°C for 5 min in the presence of 5×10^5 d.p.m. 32 P-labelled riboprobe, then hybridized at 42°C for 16 h. Following RNAase digestion, samples were electrophoresed on 6% acrylamide gels containing 7 M urea, dried and autoradiographed.

To quantify C4 and WT ER mRNAs within breast tumour samples, a standard curve was established in each assay. C4 and WT ER mRNAs (30, 125, 500 pg C4 RNA and 125, 500, 2000 pg WT ER RNA) synthesized using T7 RNA polymerase were purified on a Sephadex G-50 column and quantitated spectrophotometrically. WT ER RNA was transcribed from linearized pHEO, which contains the entire WT ER coding sequence but is missing the 3'-untranslated portion of the ER mRNA [(kindly provided by P Chambon, Strasbourg, France (Green et al, 1986)]. Full-length C4 RNA was transcribed from linearized pSK-C4 (Dotzlaw et al, 1992). Standard RNAs were analysed together in the same assay as the breast tumour mRNAs. Bands corresponding to the C4 ER mRNA and WT ER mRNA protected fragments were excised from the gel and counted after addition of 5 ml scintillant (ICN Pharmaceuticals, Inc., Irvine, CA, USA) in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA, USA). For each sample, absolute amounts of C4 and WT ER mRNA were determined from the standard curve.

Reverse transcription, PCR and triple-primer (TP) PCR

For each sample, 1 μ g of total RNA was reverse transcribed in a final volume of 15 μ l as described previously (Leygue et al, 1996a). One microlitre of the reaction mixture was taken for subsequent amplification.

The primers and PCR conditions for the long-range PCR were as previously described (Leygue et al, 1996c). The primers and PCR conditions for measuring the relative expression of exon 5-deleted and exon 7-deleted ER transcripts relative to WT ER transcripts were as previously described (Leygue et al, 1996a).

The TP-PCR conditions were similar to those previously described (Leygue et al, 1996b) with minor modifications. ERU (5'-TGTGCAATGACTATGCTTCA-3', sense, located in WT ER exon 2; 792-811, as numbered in Green et al, 1986) and ERL (5'-GCTCTCTCCTGTTTTTAT-3', antisense, located in WT ER exon 3; 940-921) primers allowed amplification of a 149-bp fragment corresponding to WT ER mRNA. The C4-specific primer (C4L, 5'-TTTCAGTCTTCAGATACCCAG-3', antisense; 1336-1315, as numbered in Dotzlaw et al, 1992) spans the only region of the C4 unique sequence that does not have any homology with repetitive LINE-1 sequences (Dotzlaw et al, 1992). ERU and C4L allowed amplification of a 536-bp fragment corresponding specifically to C4 ER variant mRNA.

PCR amplifications were performed in a final volume of 10 μ l in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM potassium chloride,

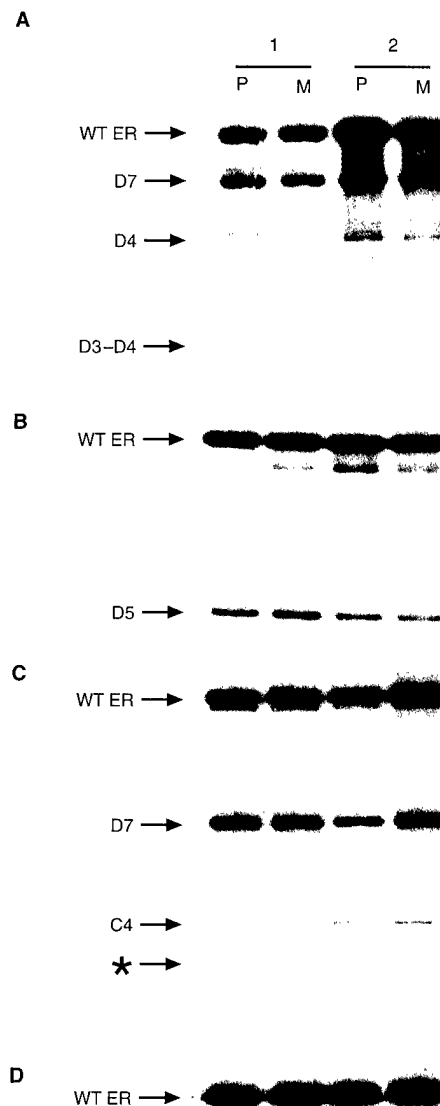


Figure 1 (A) Autoradiogram of long-range RT-PCR (Leygue et al, 1996c) results from two samples of primary breast tumours (P) and their matched concurrent lymph node metastase (M). WT ER is the expected product corresponding to the WT ER mRNA; D7 is the expected product corresponding to the exon 7-deleted ER variant mRNA; D4 is the expected product for the exon 4-deleted ER mRNA; D3-4 is the expected product for the exon 3+4-deleted ER mRNA; D4/7 is the expected product for the exon 4+7-deleted ER mRNA. (B) Autoradiogram of RT-PCR results from two samples of primary breast tumours (P) and their matched concurrent lymph node metastase (M). D5 is the expected product corresponding to the exon 5-deleted ER variant mRNA. WT ER is the expected product corresponding to the WT ER mRNA. (C) Autoradiogram of RT-PCR results from two samples of primary breast tumours (P) and their matched concurrent lymph node metastase (M). D7 is the expected product corresponding to the exon 7-deleted ER variant mRNA. WT ER is the expected product corresponding to the WT ER mRNA. (D) Autoradiogram of TP-PCR results from two samples of primary breast tumours (P) and their matched concurrent lymph node metastase (M). C4 is the expected product corresponding to the clone 4 ER variant mRNA. WT ER is the expected product corresponding to the WT ER mRNA. *Band coamplified with C4 and WT ER and shown to correspond to an exon 2-duplicated ER variant mRNA

2 mM magnesium chloride, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng μ l⁻¹ of each primer (ERU, ERL and C4L), 0.2 units of *Taq* DNA polymerase (Gibco-BRL) and 1 μ Ci of [α - 32 P] dCTP (3000 Ci mmol⁻¹, ICN Pharmaceuticals, Irvine, CA, USA). Each PCR consisted of 30 cycles (1 min at 94°C, 30 s at 60°C and

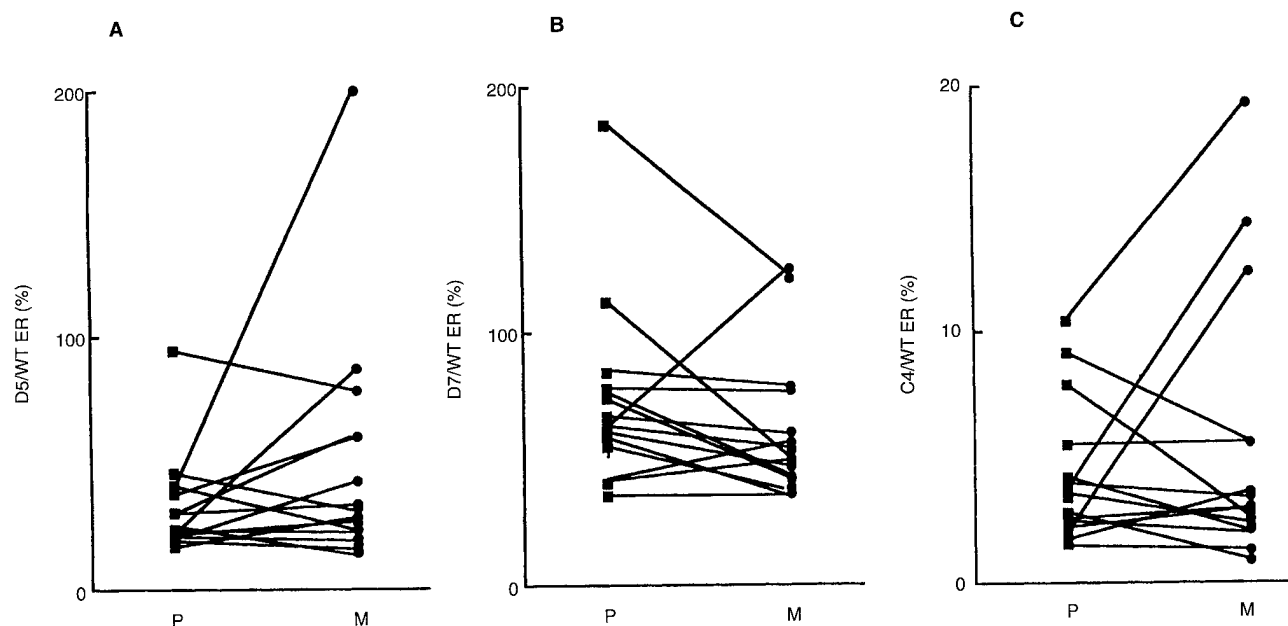


Figure 2 (A) Quantitative comparison of the relative expression of exon 5-deleted variant ER mRNA in primary (P) human breast tumours and their concurrent matched lymph node metastases (M). For each sample, the mean of three independent measures of exon 5-deleted ER relative expression expressed as a percentage of the corresponding WT ER signal was determined as described in the Materials and methods section. (B) Quantitative comparison of the relative expression of exon 7-deleted variant ER mRNA in primary (P) human breast tumours and their concurrent matched lymph node metastases (M). For each sample, the mean of three independent measures of exon 7-deleted ER relative expression expressed as a percentage of the corresponding WT ER signal was determined as described in the Materials and Methods section. (C) Quantitative comparison of the relative expression of clone 4 variant ER mRNA in primary (P) human breast tumours and their concurrent matched lymph node metastases (M). For each sample, the mean of three independent measures of clone 4 relative expression expressed as a percentage of the corresponding WT ER signal was determined as described in the Materials and Methods section

1 min at 72°C) using a thermocycler (Perkin Elmer). Four microlitres of the reaction mix was then denatured by addition of 6 µl of 80% formamide buffer and boiling before electrophoresis on 6% polyacrylamide gels containing 7 M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with two intensifying screens for 2 h.

Quantification of RT-PCR and TP-PCR

Bands corresponding to the variant ER mRNA and WT ER mRNA were excised from the gel and counted after addition of 5 ml of scintillant in a scintillation counter. The variant signal was expressed as a percentage of the WT ER signal. It should be noted that the percentage obtained reflects the relative ratio of the variant to WT ER RT-PCR product and does not provide absolute initial mRNA levels. Validation of this approach was described previously (Daffada et al, 1994, 1995; Leygue et al, 1996a, b). At least two independent PCR assays were performed for each sample in the comparison of RNAase protection assay with TP-PCR assays. For assessment of matched primary and secondary breast tumour samples, at least two and in most cases three independent PCR reactions were performed and the mean determined.

The statistical significance of differences in the relative levels of expression of any single ER mRNA variant between primary tumour and lymph node metastasis was determined using the Wilcoxon signed-rank test.

RESULTS

Determination of the pattern of exon-deleted ER variant mRNA expression

Multiple ER variant mRNAs have been shown to be expressed in any one breast tissue sample (Leygue et al, 1996a; Murphy et al, 1997a, b). To investigate the pattern of multiple exon-deleted ER variant expression between primary breast tumours and their matched lymph node metastases, a long-range RT-PCR approach was used. This approach, based on the competitive amplification of wild-type and exon-deleted ER variant cDNAs, using primers annealing within exons 1 and 8, allows the evaluation of the relative pattern of expression of all exon-deleted ER variant transcripts present in any individual sample (Leygue et al, 1996c; Fasco, 1997). Typical results are shown in Figure 1A. The pattern of deleted ER mRNA expression between any one primary tumour and its matched lymph node metastasis was conserved.

Determination of the relative expression of exon 5-deleted and exon 7-deleted ER variant mRNA expression

Using a previously validated semiquantitative PCR approach (Leygue et al, 1996a), the measurement of the relative expression of specific individual exon-deleted ER variant mRNAs was also undertaken. Specifically, the relative expressions of exon 5-deleted

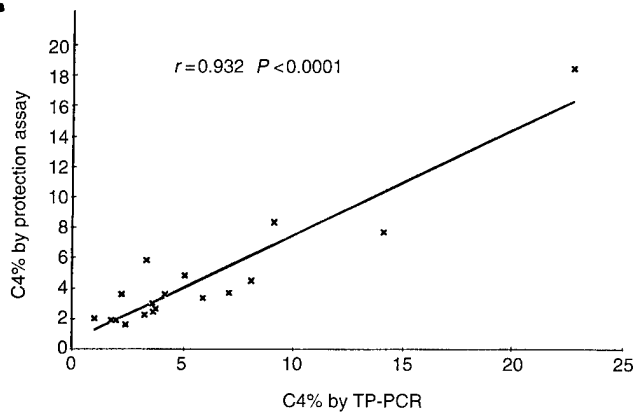


Figure 3 Linear regression analysis of clone 4 expression (expressed as a percentage of the corresponding WT ER expression) as determined by TP-PCR vs standardized RNAase protection assay in 18 human breast tumours

ER cDNA (Figure 1B) using primers in exons 4 and 6, and exon 7-deleted ER cDNA (Figure 1C), using primers in exons 5 and 8, were measured. The median value for the relative expression of the exon 5-deleted ER for the primary tumours was 23.1% (range 17.3–94.3%) and the median value for the matched lymph node metastases was 31.3% (range 14.9–200%). The scatter plot for these results is shown in Figure 2A. The median relative expression of the exon 7-deleted ER for primary tumours was 65% (range 39.3–184.9%) and the median value for the matched lymph node metastases was 52.5% (range 35.5–126%). The scatterplot of these results is shown in Figure 2B. There were no statistically significant differences in the relative expression of either exon-deleted ER mRNA between primary and concurrent metastatic tumours.

Comparison of RNAase protection assay and triple-primer PCR assay for determination of the relative expression of clone 4 truncated ER variant mRNA expression

Another frequently expressed ER variant, which would not be detected in the above assays, is the C4 ER mRNA. This variant was previously found to be significantly elevated in a group of primary breast tumours with poor prognostic features that included concurrent lymph node metastases, compared with a group of primary tumours with good prognostic variables that included lack of concurrent nodal metastases (Murphy et al, 1995). Therefore, it was relevant to determine the level of C4 ER variant expression in primary breast tumours and their matched, concurrent lymph node metastases.

In this previous study, we used RNAase protection assays to measure WT and variant ER mRNA expression (Murphy et al, 1995). However, in order to conduct this study using smaller tissue samples (in particular from nodal metastases) and to ensure a close correlation with the histological composition of the tissue, we used a previously described TP-PCR assay (Leygue et al, 1996b) to measure the relative expression of C4 ER mRNA. To facilitate comparison of the current data with our earlier study (Murphy et al, 1995), it was necessary to compare the RNAase protection assay with the TP-PCR assay, before proceeding to analyse the primary and secondary breast tumour samples for C4 mRNA expression by TP-PCR.

RNA from 25 human breast tumours, selected to represent a wide range of ER status by ligand-binding assay (Table 1), was

Table 1 C4 and WT ER mRNA expression in 25 human breast tumours, as determined by RNAase protection assay and TP-PCR

Sample no.	Ligand binding	RNAase protection		TPPCR	
	ER (fmol mg ⁻¹)	C4 (pg 10 µg ⁻¹)	WT ER (pg 10 µg ⁻¹)	C4 (%)	C4 (%)
5	0.0	ND	ND	–	1.7
3	0.4	ND	ND	–	2.6
1	0.9	ND	ND	–	3.1
24	1.2	6.2	105.1	5.9	3.3
4	1.8	ND	ND	–	3.7
23	4.5	10.0	54.3	18.4	22.7
8	5.8	ND	26.8	–	2.8
7	6.3	ND	224.6	–	3.4
2	8.7	ND	9.0	–	2.2
19	10.0	22.6	902.9	2.5	3.6
10	17.8	5.3	146.4	3.6	4.1
13	25.0	2.3	112.0	2.0	1.0
15	44.0	5.0	148.5	3.4	5.9
22	57.0	11.8	153.6	7.7	14.1
11	90.0	2.5	129.1	1.9	1.7
21	96.0	9.6	263.4	3.6	2.2
14	105.0	4.6	94.4	4.9	5.0
17	111.0	26.7	320.3	8.3	9.1
9	121.0	4.6	277.7	1.7	2.4
6	146.0	2.0	105.0	1.9	1.9
18	198.0	15.8	422.0	3.7	7.0
20	236.0	8.8	288.4	3.0	3.5
12	289.0	3.6	80.5	4.5	8.0
16	304.0	38.8	1440.8	2.7	3.7
25	311.0	83.9	3651.0	2.3	3.2

ND, not detected.

analysed in a standardized RNAase protection assay in order to determine the absolute amount of C4 and WT ER mRNAs within each sample. The signals corresponding to C4 and WT ER mRNAs were quantified as described in Materials and Methods. In each assay, known amounts of synthetic WT ER and C4 mRNAs were analysed in parallel in order to establish a standard curve allowing the determination of absolute levels of C4 and WT ER mRNAs, expressed as pg 10 µg⁻¹ RNA (Table 1). Because of the very low C4 protected fragment signal (≤ 15 d.p.m.) in seven tumours, it was not possible to determine confidently the absolute amount of C4 mRNA in these samples (not detected, ND). All C4-negative tumours by RNAase protection assay were from tumours with ER values lower than 10 fmol mg⁻¹ protein, as determined by ligand-binding assay. The absolute amounts of C4 and WT ER mRNAs in the remaining 18 tumours, as determined by RNAase protection assay, varied from 2 to 83.9 pg 10 µg⁻¹ RNA and from 9 to 3651 pg 10 µg⁻¹ RNA respectively. For each sample, the C4 mRNA signal was expressed as a percentage of WT ER mRNA signal (Table 1).

C4 ER mRNA relative expression was determined by TP-PCR within the same 25 RNA samples as described in Materials and Methods. Both C4 and WT ER cDNAs signals were detected in all 25 tumours studied, independent of their ER status as determined by ligand-binding assay. C4 and WT ER signals were quantified as described in Materials and Methods. The signal corresponding to C4 was expressed as a percentage of the WT ER signal. Table 1 presents the average of a least two independent TP-PCR experiments. Linear regression analysis (Figure 3) shows a highly significant correlation between C4 mRNA relative expression as

determined by RNAase protection assay (in the 18 tumours in which a C4 signal was detectable) and C4 mRNA relative expression determined by TP-PCR ($r = 0.932$, $P < 0.0001$). Interestingly, an additional band was also observed in most of the samples using the TP-PCR assay (see asterisk, Figure 1D). This band was identified after subcloning and sequencing to be a product of an exon 2-duplicated ER variant mRNA. The intensity of the signal obtained from this exon 2-duplicated ER band paralleled that of the WT ER band, and the co-amplification of the exon 2-duplicated ER variant mRNA using TP-PCR did not interfere with the relationship between TP-PCR and RNAase protection assay.

Determination of the relative expression of clone 4 truncated ER variant mRNA expression

The above TP-PCR assay was used to compare the relative expression of C4 and WT ER expression in the matched breast cancer samples (Figure 1D). The median relative expression of the C4 ER for the primary tumours was 3.5% (range 1.6–10.5%) and the median value for the matched lymph node metastases was 3.1% (range 1.0–19.4%). A scatterplot of the results is shown in Figure 2C. There is no statistically significant difference in the relative expression of C4 ER variant expression between primary breast tumours and their concurrent lymph node metastases by Wilcoxon rank-sum analysis. Interestingly, although not statistically significant, we found that the median level of C4 expression in ER+ PR– primary tumours, 3.7% (range 2.5–7.9%, $n = 5$), was approximately 50% higher than the median level of C4 expression in ER+ PR+ primary tumours, which was 2.4% (range 1.6–10.5%, $n = 8$). Such a trend would be consistent with our previous results in which C4 expression was higher in PR– primary breast tumours than in PR+ primary tumours.

DISCUSSION

The data presented in this study provide evidence that both the overall pattern of ER variant expression and the relative level of expression of three individual ER variants are conserved in primary breast tumours and their matched, concurrent lymph node metastases.

The observations presented in this manuscript, showing a conserved pattern and similar relative expression of ER variants between primary tumours and their concurrent lymph node metastases, would be consistent with previous observations that little change of ER status can be found between primary human breast tumours and their concurrent lymph node metastases or their distant metastases (Hahnel and Twaddle, 1985; Robertson, 1996). These findings are not inconsistent with our previously published data, which showed that the relative expression of one ER variant was significantly increased in primary tumours with poor prognostic characteristics, which included having concurrent lymph node metastases, as compared with primary tumours without concurrent lymph node metastases (Murphy et al, 1995). It should be stressed that all the primary tumours in the current study had concurrent lymph node metastases, a major feature of poor prognosis in breast cancer, and most likely resembled our previously described poor prognostic group (Murphy et al, 1995). Therefore in primary tumours that have concurrent lymph node metastases and have detectable levels of C4 ER variant as well as other variant ER mRNAs, mRNA levels do not significantly change between primary tumours and their concurrent lymph node metastases.

These data do not, however, shed any light on whether tumours with good prognostic features, as previously described (Murphy et al, 1995), that have a relatively low level of C4 ER variant mRNA subsequently develop higher levels when recurrent disease develops. Although this issue remains to be investigated, our earlier observation of higher relative C4 ER mRNA expression in PR– primary tumours compared with PR+ primary tumours appeared to be conserved in the present cohort, although the numbers were low and the difference did not reach statistical significance. As quantitative differences in the expression of several ER variants have been shown to occur in primary breast tumours compared with normal human breast tissues (Leygue et al, 1996a, b), as well as between good vs poor prognosis primary breast tumours, the current data suggest that alterations in ER variant expression and any role this may have in altered oestrogen signal transduction probably occurs early in tumorigenesis and well before the acquisition of the ability to metastasize. This is consistent with previous data supporting the concept of an early involvement of perturbations of oestrogen signal transduction and the development of hormone independence in breast tumorigenesis (Khan et al, 1994; Schmitt, 1995). It remains therefore to be determined if altered ER variant expression can predict tumour recurrence and progression in node-negative breast cancers.

To our knowledge, this study is the first to compare an already established quantitative approach, such as the RNAase protection assay, with an RT-PCR based approach in the study of ER variant mRNA expression. Earlier studies have utilized either the RNAase protection assay or RT-PCR only. Considering the potential clinical relevance of the measurement of the relative level of ER variants with respect to WT ER within human breast tissue samples and the sensitivity of an RT-PCR based approach, such a comparative study was deemed necessary. Furthermore, our data provide validation for comparing previous data obtained using a non-amplification-dependent RNAase protection assay with the current data obtained using an amplification-dependent TP-PCR assay.

The lack of sensitivity of the RNAase protection assay for a subset of tumours with very low (<10 fmol mg^{-1}) ER values by ligand-binding assay is an important limiting factor. It effectively means that, in a screening study, ER-negative tumours (<3 fmol mg^{-1} protein), as well as ER-positive tumours with ER values lower than 10 fmol mg^{-1} , as measured by ligand-binding assay, cannot be reliably assessed for C4 ER variant mRNA expression by RNAase protection assay. This, together with the relatively large amount of RNA needed to perform an RNAase protection analysis, severely limits the usefulness of a standardized RNAase protection assay in such screening studies. The low amount of starting material needed, together with the higher sensitivity observed (samples C4 ER variant negative by RNAase protection assay had detectable levels of C4 ER variant and WT ER mRNA by TP-PCR) make TP-PCR an attractive alternative to the RNAase protection assay in studies in which such factors are limiting.

In conclusion, the current investigation extends our previous studies on the relationship of ER variant expression and progression in human breast cancer. The data presented show that both the pattern and level of expression of ER variants are conserved between matched primary breast tumours and their concurrent lymph node metastases. Therefore, any alteration in ER variant expression that could be a marker of altered ER signal transduction and breast cancer progression probably occurs before breast cancer cells acquire the ability to metastasize.

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Fractionated administration of irinotecan and cisplatin for treatment of lung cancer: a phase I study

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Summary A combination chemotherapy of irinotecan (CPT-11) and cisplatin (CDDP) has been reported to be active for lung cancer. In the previous trial, however, diarrhoea and leucopenia became the major obstacle for sufficient dose escalation of CPT-11 to improve the treatment outcome. We conducted a phase I study to investigate whether the fractionated administration of CDDP and CPT-11 at escalated dose was feasible and could improve the treatment outcome. Twenty-four previously untreated patients with unresectable non-small-cell lung cancer (NSCLC) or extensive disease of small-cell lung cancer (SCLC) were eligible. Both CDDP and CPT-11 were given on days 1 and 8, and repeated every 4 weeks. The dose of CDDP was fixed at 60 mg m⁻² and given by 1-h infusion before CPT-11 administration. The starting dose of CPT-11 was 40 mg m⁻², and the dose was escalated by an increase of 10 mg m⁻². The maximally tolerated dose of CPT-11 was determined as 60 mg m⁻² because grade 4 haematological or grade 3 or 4 non-haematological toxicities developed in six patients out of 11 patients evaluated. Diarrhoea became a dose-limiting toxicity. The objective response rates were 76% for NSCLC and 100% for SCLC. The recommended dose of CPT-11 and CDDP in a phase II study will be 50 mg m⁻² and 60 mg m⁻² respectively.

Keywords: phase I study; irinotecan; cisplatin; small-cell lung cancer; non-small-cell lung cancer

Irinotecan (CPT-11) is a semisynthetic derivative of camptothecin that exerts its cytotoxic activity by inhibiting a nuclear enzyme topoisomerase (Topo) I as a novel therapeutic target (Hsiang and Liu, 1988). CPT-11 has demonstrated a remarkable anti-tumour activity for both small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) in phase II trials (Fukuoka et al, 1992; Masuda et al, 1992a). Cisplatin (CDDP), a recent key drug for treatment of lung cancer (Bonomi, 1996), has a different mechanism of action, and its overlapping toxicity with CPT-11 is minimal. Because CDDP was reported to show synergism with CPT-11 (Kudoh et al, 1993), this combination was considered to be evaluated. A phase I study of this combination for NSCLC, in which a fixed dose of CDDP (80 mg m⁻²) given on day 1 was combined with an escalating dose of CPT-11 (30–70 mg m⁻²) on days 1, 8 and 15, was reported (Masuda et al, 1992b, 1993). The maximally tolerated dose (MTD) and the recommended dose for a phase II study of CPT-11 were determined to be 70 mg m⁻² and 60 mg m⁻² respectively. In this study, a high response rate (54%) was achieved, but leucopenia and diarrhoea were dose-limiting toxicities and made further dose escalation of CPT-11 difficult (Masuda et al, 1992b, 1993). A phase II study conducted with this dose and schedule showed objective response rates of 48% for NSCLC (Nakagawa et al, 1993) and 78% for SCLC (Fujiwara et

al, 1994). Then the following dose-escalation trial was conducted by combining recombinant human granulocyte colony-stimulating factor (rhG-CSF) with the original regimen. The dose of CPT-11 could be increased up to 80 mg m⁻² (a 33% increase above the original regimen). However, diarrhoea, a dose-limiting toxicity of CPT-11, prevented further dose escalation and the objective response rate remained at 50% (Masuda et al, 1994).

The present study was planned to investigate whether the fractionated administration of both CDDP and CPT-11 on days 1 and 8 could attenuate dose-limiting toxicities and improve the treatment outcome compared with the previous trial. The primary objective of this study was to determine the MTD of CPT-11 in combination with a fixed dose of CDDP. The other objectives included evaluation of the therapeutic activity and determination of the dose-limiting toxicity of this regimen.

MATERIALS AND METHODS

Patient selection

Eligibility requirements for entry into the study were as follows: (1) histologically or cytologically proven lung cancer; (2) no prior chemotherapy, radiotherapy or surgery; (3) age of 75 years or less; (4) clinical stage of IIIA with bulky N₂, IIIB or IV for NSCLC, or extensive disease (ED) for SCLC; (5) performance status (PS) of 0–2 on the Eastern Cooperative Oncology Group (ECOG) scale (Oken et al, 1982); (6) the presence of measurable or evaluable disease; (7) adequate functions of the kidney (creatinine clearance \geq 60 ml min⁻¹), liver (ALT, AST < twice the upper limit of

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APPENDIX 5

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Prevalence of estrogen receptor variant mRNAs in human breast cancer.

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Prevalence of Estrogen Receptor Variant Messenger RNAs in Human Breast Cancer¹

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Abstract

A new approach, based on the competitive amplification of wild-type and exon-deleted estrogen receptor (ER) variant cDNAs, was used to screen 100 human breast tumors for the presence of ER variants. Already described exon 4-deleted ER mRNA was preferentially detected in tumors with lower grades ($P < 0.05$) or higher progesterone receptor levels ($P < 0.01$), whereas new ER variants, deleted in exons 2-4 or in regions within exons 3-7 were associated with higher grades ($P < 0.025$) and higher ERs ($P < 0.001$). This approach allows investigation of the expression of multiple ER variant mRNAs and may implicate them as new prognostic markers and as possible contributors to tumor progression.

Introduction

Several ER³ variant mRNAs have now been detected in both normal and cancerous breast tissues (1-11). Although it is unclear if any or all of these mRNAs are translated *in vivo*, some of the predicted ER-like proteins, lacking some functional domains (12) of the WT-ER (Fig. 1), exhibit altered functions *in vitro*. Exon 3- and exon 7-deleted variants may act as dominant negative regulators of WT-ERs (3, 6), whereas exon 5-deleted ER has ligand-independent transcriptional activity (4, 13). Changes in the balance between ER-like molecules could be involved in perturbation of the ER signaling pathway and tumor progression (14-20). Many laboratories have begun to investigate the association between the expression of individual ER variant mRNAs and the loss of hormone-dependent growth (16, 19). However, it is now apparent that several different types of variant ER transcripts and therefore predicted proteins can be expressed together (8, 9), and the validity of investigating individual variants in isolation can be questioned. Furthermore, previous analyses have depended largely on assays that focus on limited regions of the transcript and that would be unlikely to detect more than one modification per individual variant mRNA. However, it is now clear that more than one modification can occur in variant transcripts (17). Thus, signals attributed to the exon 7-deleted ER variant mRNA detected with reverse transcription-PCR using primers in exons 5 and 8 (9) or with RNase protection assays with probes covering the exon 6-8 junction (20)

may also include contributions from a variant deleted in both exons 4 and 7 recently identified by Madsen *et al.* (17). Nevertheless, these molecules may result in quite different proteins which differ in activity and modulate differentially the ER signaling pathway. Moreover, because of the lack of an approach to investigate qualitatively and quantitatively the representation of total ER variant mRNAs within any one given sample, it becomes difficult to evaluate those variants potentially important *in vivo* either as prognostic markers or as possible contributors to tumor progression. The purpose of this study was to develop a strategy that would allow the investigation of known and unknown exon-deleted or -inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants among themselves and with respect to the WT-ER transcript. The approach used is depicted in Fig. 1. cDNAs corresponding to all exon-deleted ER variants identified to date can be amplified along with the WT-ER mRNA using primers annealing with exon 1 (1/8U) and exon 8 (1/8L) sequences. We assumed that a competitive amplification could therefore occur among all exon-deleted or -inserted ER variant transcripts that would depend on their initial relative representation, the detection of bands corresponding to specific ER variants reflecting the balance between ER variant mRNA species within the sample. Since it is likely that alterations in the coding sequences could be translated into ER-like proteins with altered functions, we have for practical reasons confined our approach to the coding region only. This approach was tested in this pilot study to determine the incidence of ER variants in a set of 100 breast tumors that were selected to represent a wide range of breast cancers with respect to ER and PR levels, size, grade, and axillary nodal status.

Materials and Methods

Human Breast Tissues and Cell Line. All human breast tumor specimens were obtained from the Manitoba Breast Tumor Bank. Tumors (100 cases) were chosen to represent a variety of tumor characteristics represented in the breast tumor population collected in the Manitoba Breast Tumor Bank. Thirty tumors were ER negative (ER < 3 fmol/mg protein), with PR values ranging from 0 to 25 fmol/mg protein, as measured using the ligand-binding assay. Seventy tumors were ER positive (ER ranging from 3.6 to 386 fmol/mg protein), with PR values ranging from 0 to 297 fmol/mg protein. These tumors also spanned a wide range of grades (from 4 to 9), determined using the Nottingham grading system (21), size (ranging from 1 to 6.3 cm), and nodal status (absence or presence of axillary nodes). T-47D-5 cells, which are known to express different ER variant mRNAs (11, 18), were kindly provided by Dr. R. L. Sutherland (Garvan Institute for Medical Research, Sydney, Australia). Total RNA was extracted and reverse transcribed in a final volume of 15 μ l as described previously (11).

Primers and PCR Conditions. The primers used consisted of 1/8U primer (5'-TGCCCTACTACCTGGAGAACG-3', sense; located in WT-ER exon 1; 615-637) and 1/8L primer (5'-GCCTCCCCCGTGATGTAA-3', antisense; located in WT-ER exon 8; 1995-1978). Nucleotide positions given correspond to published sequences of ER cDNA (22). PCR amplifications were performed, and PCR products were analyzed as described previously (11), with minor modifications. Briefly, 1 μ l of reverse transcriptase mixture was amplified in a final volume of 10 μ l in the presence of 10 nM [α -³²P] dCTP, 4 ng/ μ l of each primer, and 1 unit

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³ The abbreviations used are: ER, estrogen receptor; WT, wild type; PR, progesterone receptor; D2-, D3-, D4-, D5-, D7-ER, variant mRNA deleted in exons 2, 3, 4, 5, and 7, respectively; D3-4-, D2-3-ER, variant mRNA deleted in both exons 3 and 4 and in exons 2 and 3, respectively; D4/7-ER, variant mRNA deleted in both exons 4 and 7; D2-3/7-ER, variant mRNA deleted in exons 2, 3, and 7; D2-3-4-ER, variant mRNA deleted in exons 2, 3, and 4; D-3-7-ER, variant mRNA deleted in sequences within exon 3 to within exon 7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

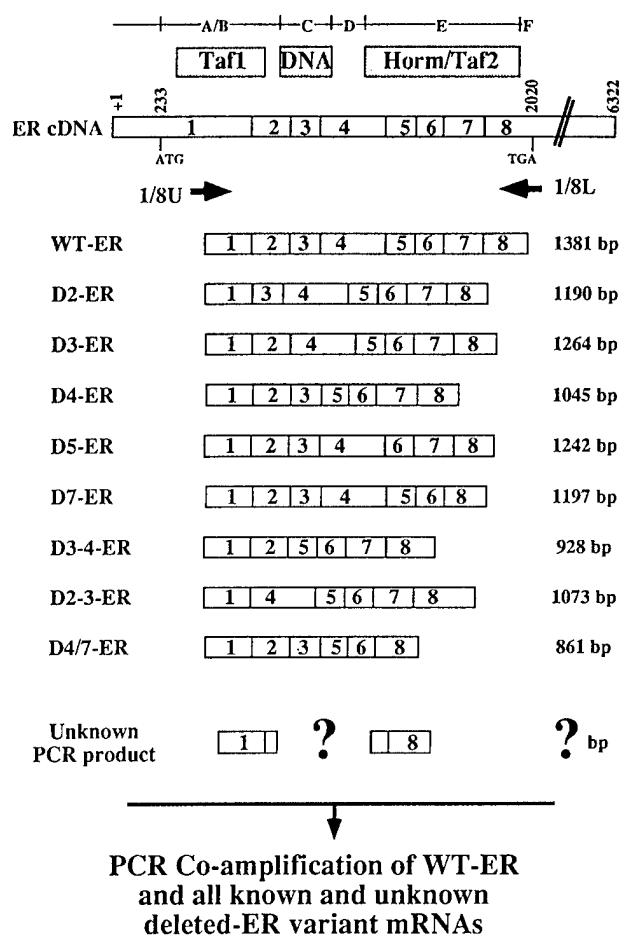


Fig. 1. Schematic representation of WT-ER cDNA and primers allowing coamplification of most of the described exon-deleted ER variants: ER cDNA contains eight different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in *trans*-activating function (Taf1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another *trans*-activating function (Taf2). 1/8U and 1/8L primers allow amplification of the 1381-bp fragment corresponding to WT-ER mRNA. Coamplification of all possible exon-deleted or -inserted variants which contain exon 1 and 8 sequences can occur. Amplification of the previously described ER variant mRNAs deleted in exon 2 (D2-ER), exon 3 (D3-ER), exon 4 (D4-ER), exon 5 (D5-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER), exons 4 and 7 (D4/7-ER) would generate 1190-bp, 1264-bp, 1045-bp, 1242-bp, 1197-bp, 928-bp, 1073-bp, and 861-bp fragments, respectively.

of *Taq* DNA polymerase. Each PCR consisted of 40 cycles (1 min at 60°C, 2 min at 72°C, and 1 min at 94°C). PCR products were then separated on 3.5% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. To control for errors in the input of cDNA used in PCR reactions, amplification of the ubiquitous GAPDH cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (11). All PCR products were subcloned and sequenced as described previously (11).

RNA Dilution Experiments. Plasmids that contained PCR products subsequently identified as fragments corresponding to exons 3- and 4-deleted ER variant (D3-4-ER) and to variant deleted in exons 2, 3, and 7 (D2-3/7-ER) were linearized with *Bam*HI and gel purified as described previously (11). Corresponding sense RNAs were synthesized using Riboprobe Systems (Promega, Madison, WI) according to the manufacturer's instructions. One μ g of total RNA from T-47D-5 cells was mixed with various amounts of synthetic D2-3/7-ER (ranging from 5 ng to 50 fg) or D3-4-ER RNA (50 fg). These spiked RNA samples were then reverse transcribed and amplified using 1/8U and 1/8L primers as described above.

Statistical Analysis. Each individual tumor sample was analyzed in at least three independent assays. Only bands reproducibly observed in three experiments were considered. The presence of a specific band in a tumor sample was scored only if its signal intensity placed it among the four strongest signals (as assessed

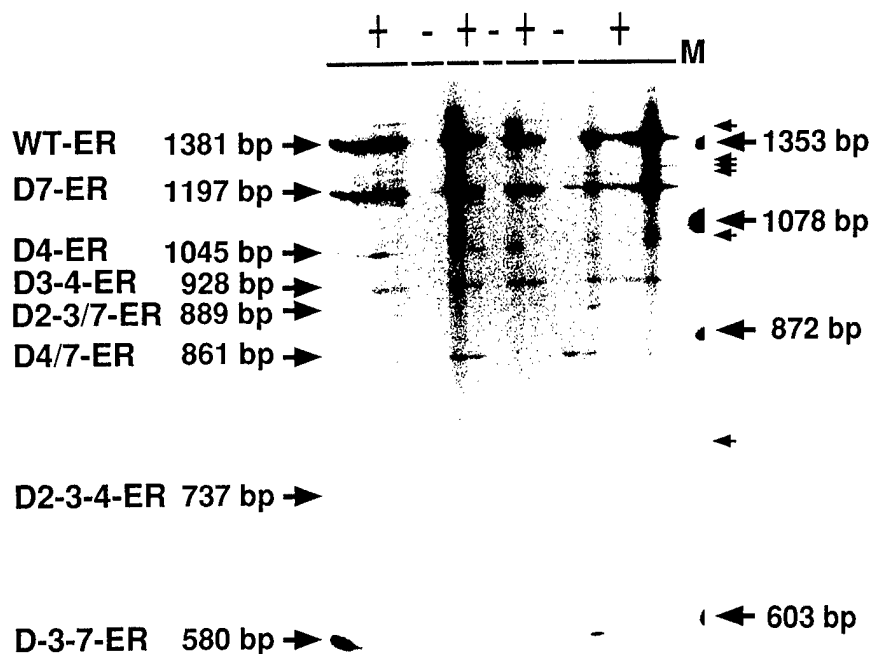
by subjective visualization) observed in the corresponding lane. The tumor group in which the band corresponding to the WT-ER mRNA was detected (68 cases) presented the following characteristics: ER level ranging from 0 to 386 fmol/mg protein (average, 111 fmol/mg protein) and PR level ranging from 0 to 297 fmol/mg protein (average, 73 fmol/mg protein). For the purpose of analysis, this group was divided into two subgroups presenting ER, PR, or a grade above or below a point defined as the average of the ER value, PR value, or grade observed within the group. Possible associations between the detection of a particular variant and one particular subgroup were tested using either the χ^2 test, including Yates' correction when the estimated frequency was at least equal to 5, or the Fisher exact test (two tailed) in other cases.

Results

Coamplification of WT-ER mRNA and Deleted Variant mRNAs in Breast Tumor Samples. On the basis of the assumption that coamplification of WT-ER mRNA and variant ER mRNAs could effectively occur and therefore allow identification of the frequency and relative expression of variants in breast tumor tissues, 100 breast tumors were selected for analysis that represented a wide range of ER and PR levels, as measured by the ligand-binding assay, grade, nodal status, and size. Total RNA was extracted from each tumor sample and reverse transcribed. PCR was then performed using primers annealing with exon 1 and exon 8 sequences. Fig. 2 shows typical results obtained. Many different PCR products were observed in each of 70 ER-positive tumors but only in 3 of 30 ER-negative tumors. This difference did not result from variable input of cDNA, since similar signals were obtained in all samples after amplification of the housekeeping GAPDH cDNA (data not shown). Two bands that migrated with the apparent sizes of 1381 and 1197 bp were observed in most of the signal-positive tumors. These bands were detectable in 68 and 63 cases, respectively. Following subcloning and sequencing, these bands were shown to correspond to the WT-ER and an exon 7-deleted ER (D7-ER) variant mRNA, respectively. Six other bands that migrated at the apparent sizes of 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were consistently detected within the set of tumors studied, but at an apparently lower frequency. They were observed in 19, 8, 6, 11, 6, and 20 tumors and were found to correspond to ER variant mRNAs deleted in exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3, and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2-4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. Sequences of all of these variants, except D-3-7-ER variant, showed a perfect junction between exons surrounding the deletion area (data not shown). A 801-bp deletion was observed in the D-3-7-ER variant from nucleotides 931 to 1729 (22) located within exon 3 and exon 7, respectively. It should be stressed that some bands, either not consistently observed or specific for less than three tumors, have not yet been assessed further in this study.

Detection of a Particular Variant Depends on Its Initial Representation within the ER mRNAs Population. To determine whether the detection of a variant depended on its initial representation within the ER-like mRNA population, the balance of ER-deleted variants was artificially changed in favor of particular variants. Various amounts of synthetic RNAs corresponding to the D3-4-ER and D2-3/7-ER PCR products were added to total RNA extracted from T-47D-5 breast cancer cells. These RNA preparations were reverse transcribed and subsequently analyzed with PCR using 1/8U and 1/8L primers (Fig. 3). Bands corresponding to WT-ER, D7-ER, D4-ER, and D-3-7-ER were initially detected in T-47D-5. The addition of synthetic D2-3/7-ER RNA, which increased its ability to compete for the binding of 1/8U and 1/8L primers during the PCR reaction, drastically decreased signals corresponding to the initially detectable endogenous variants. The extinction of these signals was directly related to the concentration of the synthetic RNA added. The Addition

Fig. 2. Coamplification of WT-ER and deleted variant mRNAs in breast tumor samples: total RNA extracted from different ER-positive (+) and ER-negative (-) breast tumors was reverse transcribed and PCR amplified as described in "Materials and Methods" using 1/8U and 1/SL primers. Radioactive PCR products were separated on a 3.5% acrylamide gel and visualized using autoradiography. To each lane corresponds a unique tumor. Bands reproducibly obtained within the set of tumors studied and that migrated at 1381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were identified as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3, and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2-4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. PCR products indicated by small arrows, barely detectable within the tumor population, *i.e.*, present in less than or equal to three particular tumors, have not yet been identified. M, molecular weight marker (ϕ X174; Life Technologies, Inc., Grand Island, NY).



of two synthetic RNAs simultaneously resulted in the increased representation of two expected bands.

Detection of Particular Variants May Be Associated with Tumor Characteristics. Detection of ER variants using the approach described here appeared to depend on the initial relative ratio of expression between ER-like mRNAs. It was therefore of interest to search for possible associations between the detection of particular variants and other tumor characteristics. The detection of a specific

band in a sample was defined here as its presence as one of the four main signals observed in the corresponding lane. The frequency of detection of each ER variant mRNA within tumors also expressing a detectable WT-ER band is presented Table 1. Using the mean ER, PR, and grade values as cutoff points for statistical analysis, we found that D-3-7-ER and D2-3-4-ER variants were preferentially detected in the subgroup with higher ER ($P < 0.001$) and higher grade ($P < 0.025$), respectively. D4-ER variant was more frequently observed in tumors of lower grade ($P < 0.05$) or with higher PR levels ($P < 0.01$).

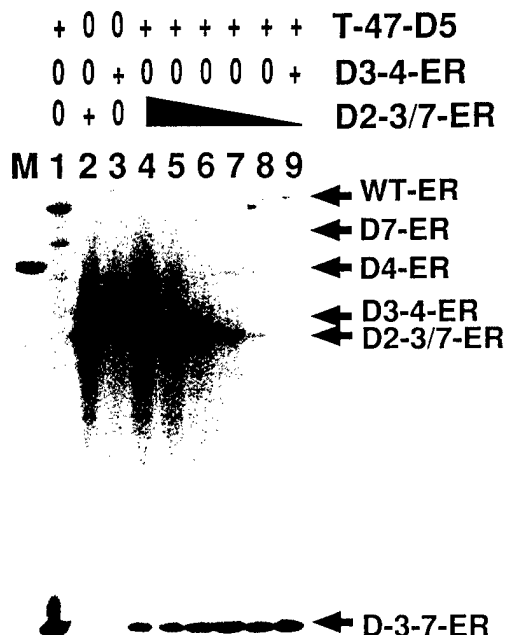


Fig. 3. Coamplification of WT-ER and deleted variant mRNAs after artificial modification of the balance between ER-like mRNAs: one μ g of total RNA from T-47D-5 cells alone (Lane 1) or mixed with various amounts of synthetic D2-3/7-ER (5 ng, 500 pg, 50 pg, 5 pg, 500 fg, and 50 fg; Lanes 2, 3, 4, 5, 6, 7, 8, and 9, respectively) and D3-4-ER (50 fg, Lane 9) RNAs. These spiked RNAs were then reverse transcribed and amplified as described in "Material and Methods." PCR products were separated on 3.5% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gel was dried and autoradiographed for 18 h. D2-3/7-ER and D3-4-ER RNAs alone (5 ng) were similarly analyzed (Lanes 2 and 3, respectively). M, molecular weight marker.

Discussion

We have used a new approach based on the competitive coamplification of WT-ER and exon-deleted or -inserted ER variant mRNAs to examine the overall expression of these two types of ER variants which encompass the majority of ER variant mRNAs thus far identified (23). Although another distinct group of variants, the truncated ER variants that include the widely expressed ER clone 4 variant (5, 18), cannot be assessed with this analysis, the strategy allows a broad investigation of the ER-like population and the integrity of the entire coding region within this species, without focusing on particular regions. This has enabled us to confirm the existence of four variants already described by others, *e.g.*, exon 7-deleted ER variant (3, 16), exon 4-deleted ER variant (7), exon 3-4-deleted ER variant (9), and a variant deleted in both exons 4 and 7 (17). Beyond these, three new variants were identified. Two of them, deleted in exons 2, 3, and 7 or exons 2-4, correspond to the usual exon-deleted ER variant pattern, *i.e.*, containing a perfect deletion of exon sequences. The third one contained part of exon 3 attached to a sequence beginning inside the seventh exon. It should be noted that very recently, Daffada and Dowsett (24) identified an ER variant presenting a similar pattern of intra-exon deletion between exons 4 and 7. Furthermore, we have been able to detect ER variant mRNA deleted in both exons 4 and 7 for the first time in multiple clinical material, supporting the potential relevance of such a variant *in vivo*. The function of the putative encoded protein which lacks a nuclear localization signal, all of the hinge domain, and is C-terminal truncated remains to be determined.

Using different RNA preparations, we showed that the detection of

Table 1 Frequency of detection of ER variant mRNAs within 68 human breast tumors also expressing detectable WT-ER mRNA

	Tumors expressing WT-ER	No. of tumors expressing detectable ER variant mRNAs					
		D7-ER	D4-ER	D3-4-ER	D2-3/7-ER	D4/7-ER	D2-3-4-ER
ER < 111 (fmol/mg protein)	38	34	8	6	4	7	3
ER > 111 (fmol/mg protein)	30	27	9	2	2	3	2
PR < 73 (fmol/mg protein)	41	35	5	7	5	6	4
PR > 73 (fmol/mg protein)	27	26	12	1	1	4	1
4 ≤ grade ≤ 6	35	33	13	4	3	5	0
7 ≤ grade ≤ 9	33	28	4	4	3	5	5
							9

WT and variant ER mRNAs were detected after co-amplification as described in "Materials and Methods."

^a P values calculated using the χ^2 test with Yates' correction.

^b P value calculated using the Fisher exact test (two tailed).

a variant depended on its initial representation within the ER-like mRNA population. The absence of a prominent signal corresponding to any particular variant could therefore result from its low relative representation. This could explain why variants deleted in either exon 3 or exon 5 were undetectable using our criteria and this approach, although their presence was confirmed by specific PCR amplification in some of the same tumors studied.⁴ These variants may also correspond to infrequent or poorly represented ER-like mRNAs and therefore PCR products that we have not yet identified. On the other hand, the detection of any particular ER variant mRNA within a tumor sample can result from its overexpression or a change in the balance between all ER variant mRNAs. Using this approach, it is therefore possible to investigate the relative proportion of ER variant mRNAs, and also to compare breast samples regarding the relative expression of their ER-like mRNAs.

The set of tumors analyzed in this pilot study was chosen to obtain the widest qualitative representation of important breast tumor characteristics more than to establish statistical associations. The tumor population contained very different tumors spread over a wide range of ER and PR levels, size, grade, and nodal status. It was possible however to establish that detection of particular variants may be correlated with already known prognostic markers. It is interesting to note that the exon 4-deleted variant is associated in this study group with two different markers of good prognosis, *i.e.*, high PR and lower grade. This variant, initially described in breast cancer cell lines (7) and subsequently *in vivo* in several normal and tumor tissues (9, 10), is expected to encode an ER-like protein lacking most of the hinge domain, which includes an important nuclear localization signal and a part of the hormone binding domain. It might therefore have a cellular distribution and estrogen-binding affinity different from that of the WT-ER. Furthermore, the altered structure of this protein may lead to altered transcriptional activities.

The use of this approach to study a larger set of samples would allow the establishment of a typical pattern of ER variant mRNA expression for each type of tumor. Comparison of such patterns along with the subsequent analysis of the specifically detected transcripts could lead to the discovery of new prognostic factors and the identification of new contributors to tumor progression.

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⁴ E. Leygue, unpublished data.

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APPENDIX 6

Coutts AS, **Leygue E**, and Murphy LC

Variant estrogen receptor alpha messenger RNA in hormone independent
human breast cancer cells.

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**Variant estrogen receptor- α messenger RNA expression in hormone-independent human
breast cancer cells.**

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Abbreviated title: Variant estrogen-receptor- α and estrogen-independence.

Key words: Estrogen receptor, estrogen-independence, variant, breast cancer, exon deletion.

Abstract.

T5-PRF cells are insensitive to the growth-stimulatory effects of estrogen while still retaining expression of ER- α . In the apparent absence of ligand T5-PRF cells have a 3.6 ± 0.5 fold increased basal ER- α activity and elevated basal progesterone receptor (PR) levels compared to the parent, estrogen sensitive, T5 cells. Long range ER- α reverse transcription-polymerase chain reaction (RT-PCR) was performed to characterize variant ER- α mRNA expression in the two cell lines. An increased relative expression of an exon 3+4 deleted ER- α mRNA variant was found in T5-PRF. Recombinant expression of this ER- α variant resulted in significantly increased estrogen-responsiveness, as well as a trend to increased basal ligand independent activity when expressed with wild-type ER- α in ER negative cell lines, as well as significantly increasing both ligand-independent and estrogen-induced ER- α transcriptional activity when expressed in parental T5 cells. These results suggest a role for altered variant ER- α in ligand-independent activation of ER- α which may contribute to hormone-independence in breast tumours.

Introduction

Breast cancer is a hormonally responsive cancer and hormones, including estrogen, are required for breast cancer growth (Dickson, 1991). Estrogens promote the growth of human breast cancer, and as such, most endocrine therapies are aimed at blocking the growth promoting effects of estrogen (e.g., antiestrogen such as tamoxifen). Breast cancers are classified according to their requirement for proliferation as being either hormone-dependent or hormone-independent, based ultimately on the response to endocrine therapy of metastatic disease (Nandi *et al.*, 1995). The level of estrogen receptor-alpha (ER- α) in human breast cancer (HBC) is used as a marker not only of potential therapeutic response to endocrine therapy, but is a marker of prognosis and survival (Merkel & Osborne, 1989).

The evolution of breast cancer into an estrogen-independent growth phenotype is thought to be an important step in the progression of breast cancer to hormone-independence and endocrine therapy resistance (Clarke *et al.*, 1990; Leonessa *et al.*, 1992). Understanding the factors that contribute to the development of a hormone-independent phenotype is of major importance in terms of breast cancer therapeutics. Resistance to endocrine therapies may be due to a number of factors. In some cases, hormone-independence and resistance can occur due to loss of ER expression, but most tumours that have developed resistance to endocrine therapy remain receptor positive (Horwitz, 1993).

Several breast cancer cell lines in culture also require estrogen for growth and long-term culture in estrogen-depleted conditions can result in cells becoming apparently independent of the requirement for estrogen for growth. Indeed, the development of estrogen-independent growth in human breast cancer is thought to be one of the initial steps in the progression to hormone-independence and resistance to endocrine therapies (Leonessa *et al.*, 1992). However, the mechanisms responsible for the development of estrogen-independence in the presence of continued expression of ER- α are poorly understood. In order to address this we have developed a breast cancer cell model of apparent estrogen independence (Coutts *et al.*, 1996). T5 human breast cancer cells are ER- α positive and estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 cells by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen seen in the parent cell line while still retaining expression of

the ER- α (Coutts *et al.*, 1996). However, these cells remain sensitive to the growth inhibitory effects of 4-hydroxy-tamoxifen (OT) and ICI 164,384 (ICI), although they have reduced sensitivity to ICI compared to the parent T5 cells (Coutts *et al.*, 1996).

We have investigated the ligand-dependent and -independent activity of the endogenous ER- α as well as the pattern and potential function of ER- α variant expression in T5 and T5-PRF human breast cancer cells.

Materials and Methods

Materials. (^{32}P)dCTP and (^{35}S)ATP were purchased from ICN (St-Laurent, Quebec). Dulbecco's Minimal Essential Medium (DMEM) powder and fetal bovine serum were purchased from GIBCO/BRL (Burlington, Ontario). Horse serum and EGF were purchased from UBI (Lake Placid, New York). All other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario). Cholera toxin, 4-hydroxy-tamoxifen, estradiol-17 β and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). (^{14}C)-chloramphenicol, (^3H)-R5020 (88.7 Ci/mmol), (^{35}S)-methionine and R5020 were obtained from NEN (Lachaire, Quebec). ICI 164,384 was a gift from ICI (Macclesfield, Cheshire).

Cells and Cell Culture. T5 cells, previously called T-47D5, were originally thought to be a T-47D subline, however, DNA fingerprinting analysis showed that they were an MCF-7 subline (Watts *et al.*, 1992). T5 and MDA-MB-231 human breast cancer cells were routinely cultured in DMEM containing 5% vol/vol fetal calf serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin. T5-PRF cells were routinely cultured in phenol red-free DMEM supplemented with 5% vol/vol twice charcoal dextran stripped fetal calf serum and 1% wt/vol glucose, glutamine, and penicillin-streptomycin (PRF/DMEM). MCF10A1 human breast epithelial cells (Karmanos Cancer Institute, Michigan) were grown routinely in DMEM containing 5% vol/vol horse serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin, 0.1 $\mu\text{g/ml}$ cholera toxin, 20 ng/ml hEGF, 10.4 $\mu\text{g/ml}$ bovine insulin and 1 μM hydrocortisone (DMEM-special). Transient transfections and steroid receptor assays were performed in PRF/DMEM. Transient transfections using MCF10A1 cells were performed in phenol red-free DMEM containing 5% vol/vol charcoal-stripped horse serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin (PRF/DMEM-hs) and cells were passaged once prior to transfection in phenol red-free DMEM containing 5% vol/vol charcoal-stripped horse serum, 1% wt/vol glucose, glutamine, penicillin-streptomycin, 0.1 $\mu\text{g/ml}$ cholera toxin, 20 ng/ml hEGF, 10.4 $\mu\text{g/ml}$ bovine insulin and 1 μM hydrocortisone (PRF/DMEM-special).

Progesterone Receptor Assays. PR assays were performed using whole cell ligand binding assays as previously described (Murphy & Dotzlaw, 1989a). (^3H)-R5020 and (^3H)-R5020 plus 100 fold molar excess unlabelled R5020 were used to determine PR total and nonspecific binding, respectively. All assays were performed in the presence of 100 nM dexamethasone to prevent binding of R5020 to the glucocorticoid receptor.

Transient transfections and CAT assays. T5, T5-PRF and MDA-MB-231 cells were passaged once in PRF/DMEM and set up in 100 mm diameter dishes at 0.5×10^6 cells per dish in PRF/DMEM the day before transfection. MCF10A1 cells were passaged once in PRF/DMEM-special and set up in 100 mm diameter dishes at 2×10^6 cells per dish in PRF/DMEM-special two days before transfection. The following day the medium was changed to PRF/DMEM-hs and cells were transfected the following day, using the calcium phosphate/glycerol shock method (Graham & Eb, 1973) using an equal volume 2 x BBS buffer (50mM BES, 280mM NaCl, 1.5mM Na_2HPO_4 pH 6.95), followed by a 2 minute glycerol shock (20% vol/vol). Cells were washed twice with PBS and given fresh medium plus or minus 10 nM estradiol-17 β (E2), 10nM estradiol-17 β plus 1 μM ICI 164,384 or 1 μM ICI 164,384 alone. After 24h of treatment, the cells were harvested, cell extracts prepared and chloramphenicol acetyltransferase (CAT) activity measured (Kingston, 1989). Transfection efficiency was determined by cotransfection of pCH110 (β -galactosidase expression vector, Pharmacia) and assay of β -galactosidase activity (Rosenthal, 1987) was used to correct volumes of cell extracts used for CAT assay. Therefore all results are expressed as % CAT activity or Fold CAT activity after correction for transfection efficiency. T5 and T5-PRF cells were transfected with 5 μg of ERE-tk-CAT (Seiler-Tuyns *et al.*, 1986), to determine ER- α transcriptional activity, along with 5 μg pCH110. In the experiments where activity of d3/4 was examined in T5 cells, transfections were performed using 5 μg ERE-tk-CAT, 5 μg pCH110 plus or minus d3/4 expression vector (0.1-1 pmol) or vector DNA alone. MDA-MB-231 and MCF10A1 cells were transfected with 5 μg ERE-tkCAT, 5 μg pCH110, plus or minus 0.5 pmol HEGO (wild-type ER- α expression vector, kindly provided by Dr P. Chambon) with increasing amounts (0.5-2 pmol) of d3/4 expression vector or vector DNA alone.

Long-Range ER- α RT-PCR. Total RNA was extracted (Trizol reagent, GibcoBRL, Grand Island, NY) and reverse transcribed as described previously (Leygue *et al.*, 1996c). The primer pair used consisted of 1/8U primer (5'-TGCCCTACTACCTGGAGAACG-3', sense; located in WT-ER- α exon 1; nucleotides 615-637) and 1/8L primer (5'-GCCTCCCCCGTGATGTAA-3', antisense; located in WT-ER- α exon 8; nucleotides 1995-1978). Nucleotide positions given correspond to published sequences of the human ER- α cDNA (Green *et al.*, 1986). PCR amplifications were performed as previously described (Leygue *et al.*, 1996a). PCR products were separated on 3.5% polyacrylamide gels containing 7M urea, gels were dried and labelled products visualized by

autoradiography. PCR products were subcloned and sequenced as previously described (Leygue *et al.*, 1996c).

Construction of variant ER- α expression vector. The RT-PCR product corresponding to the exon 3/4 deleted ER- α cDNA was cloned into the TA cloning vector (Invitrogen TA cloning kit). *Stu*I digestion of this plasmid released an exon 3/4 deleted fragment which was used to replace the corresponding region of wild-type ER- α from pOR8 (Tora *et al.*, 1989a) (contains a glycine to valine point mutation at amino acid 400). *Stu*I sites are in exon 2 and 7 of wild-type ER- α and the subcloned d3/4 PCR fragment resulted in a correction to the wild-type sequence of glycine at amino acid 400 (in exon 5 in pOR8). The full length *Eco*R I ER- α fragment from HEGO (an expression plasmid containing wild type ER- α coding region cloned into the eukaryotic expression vector pSG5, a gift from Dr. P. Chambon, Strasbourg, France (Tora *et al.*, 1989a)) was then excised and replaced with the corresponding fragment from pOR8 containing the exon 3/4 deleted ER- α cDNA. The identity of the expression plasmid containing the exon 3/4 deleted ER- α (called d3/4) was confirmed by restriction enzyme digest and sequence analysis.

***In vitro* transcription and translation.** *In vitro* transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega, Madison, WI). Reactions were performed according to the manufacturer's instructions.

Western blotting and immune detection. Whole cell extracts (dissolved in 8 M urea) were analysed using 10% SDS-PAGE with a 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method (Laemmli, 1970). Gels were transferred to nitrocellulose using CAPS transfer buffer (10 mM CAPS, pH 11, 20% methanol) and transferred for 1 hr at 120 V at 4°C. Blots were blocked for 1 hr at room temperature in 5% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Blots were incubated with either: ER- α specific primary antibody, H226, (a generous gift from Dr G. Greene, University of Chicago, IL which recognizes an epitope in exon 1/exon 2 region of the wild-type ER- α) or the ER- α specific antibody, AER 308, (Neomarkers, CA, which recognizes an epitope in exon 4 of the wild-type ER- α), overnight at 4°C in 1% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Blots were then incubated with the appropriate horse radish peroxidase conjugated secondary antibody for 1 hr at room temperature in 1% skim milk/Tris-buffered saline containing 0.5% Tween-20. Detection was carried out using the ECL detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, England).

Statistical Analysis. Statistical analyses of ER- α transcriptional activity and PR levels were performed using paired Student's t-test. Statistical analysis on the effects of d3/4 on ERE-tk-CAT activity in MDA-MB-231 and MCF10A1 cells was done by ANOVA (two-tailed). Statistical analysis on the effects of d3/4 on ERE-tk-CAT activity in T5 cells was done by ANOVA (two-tailed) after log transformation of the data. Statistical analyses were performed with the help of M. Cheang, University of Manitoba, Biostatistical Consulting Unit.

Results

Apparent ligand-independent (basal) activity of ER- α is increased in T5-PRF cells.

Previously, we have shown that T5-PRF cells (derived by long term passage of parental T5 cells in estrogen depleted medium) are no longer growth responsive to estrogen in culture, but still retain expression of ER- α (Coutts *et al.*, 1996). To investigate further the mechanism(s) responsible for estrogen-nonresponsiveness in these cells, ER- α transcriptional activity was examined by transient transfection assays using an estrogen-responsive reporter gene. The histogram in Figure 1A represents the fold difference in chloramphenicol acetyl transferase (CAT) activity between T5 and T5-PRF cells. As expected, estrogen treatment increases CAT activity in T5 and to a lesser extent in T5-PRF cells, while the antiestrogen ICI 164,384 (ICI) inhibits the estrogen induced transcriptional activity of the ER- α in both cell lines. In the absence of added estrogen there is a low basal ER- α activity in parental T5 cells, however in the estrogen-nonresponsive T5-PRF cells, the basal ER- α activity was 3.6 ± 0.5 (mean \pm sem, n=7) fold higher than that seen in T5 cells (p<0.05). Consistent with the increased basal CAT activity in T5-PRF cells being mediated by ER- α , treating cells under basal conditions with ICI alone, almost completely abolished the basal transcriptional activity measured (Figure 1B).

Progesterone receptor levels are elevated in T5-PRF cells.

PR expression is a marker of ER- α activity (Horwitz & McGuire, 1978), therefore we examined PR levels to determine if the increase in basal ER- α activity in T5-PRF cells was reflected in an endogenous estrogen-responsive gene. Under basal (i.e. no added estrogen) conditions the T5-PRF cells have significantly higher PR levels (~3 fold) than the parent T5 cells passaged twice in estrogen depleted medium (PRF/DMEM as defined in Methods) before receptor assays (464 ± 20 fmol/ 10^6 cells vs 148 ± 40 fmol/ 10^6 cells, mean \pm sem, n=3, see Figure 2). Previously, we had shown that T5-PRF cells retained expression of ER- α which was approximately 50% of the levels seen in the parent T5 cells, as determined by ligand binding assay (Coutts *et al.*, 1996). Since we observed increased basal activity from both an estrogen-responsive reporter gene and an endogenous estrogen-responsive gene (PR) in T5-PRF cells despite a decreased level of endogenous ligand-binding ER, we reasoned that the intrinsic activity of the wild-type ER in these cells was increased or some ER-like activity existed that was not detectable by ligand binding experiments.

Expression of a variant ER- α mRNA deleted in exons 3 and 4 is increased in T5-PRF cells.

Alterations in the structure or presence of variant forms of the ER- α with ligand-independent activity could be one mechanism for our observed results. Long-range ER- α RT-PCR analysis (Leygue *et al.*, 1996a) was performed on RNA isolated from T5 and T5-PRF cells to examine the pattern of deleted variant ER- α mRNA expression. RT-PCR analysis was performed, using a primer pair specific for exons 1 and 8 of wild-type human ER- α sequence, allowing detection of any variant ER- α mRNA species containing both exons 1 and 8 of wild-type ER- α sequence. Figure 3 shows the PCR products obtained and the presence of a 928 bp band whose relative expression is markedly increased in T5-PRF estrogen-independent cells. To confirm the identity of this variant, the cDNA corresponding to the 928 bp band was subcloned and sequenced. The nucleotide sequence of the cDNA was found to represent a variant ER- α mRNA containing a deletion of both exons 3 and 4. The exon 3 and 4 deleted ER- α (d3/4) is in frame and is predicted to encode a protein of 443 amino acid residues with a predicted molecular mass of ~49 kDa. This putative ER- α -like protein would be missing the second zinc finger of the ER- α DNA binding domain, the hinge region and part of the ligand binding domain.

The exon 3/4 deleted ER- α -like protein increases basal and estrogen-regulated wild-type ER- α transcriptional activity.

To address the potential function of this variant ER- α mRNA eukaryotic expression vectors containing d3/4 cDNA were constructed and shown to express a protein of the appropriate size that was recognized by the ER- α antibody H226 that recognizes an epitope encoded in exon 1/2 (A/B region) of wild-type ER- α (data not shown). Using an antibody that recognizes an epitope encoded in exon 4 of the wild-type ER- α the band corresponding to the d3/4 protein is not seen, while wild-type ER- α is still detected (data not shown). Ligand binding analysis of the *in vitro* translated d3/4 protein showed little or no ability to specifically bind radiolabelled estradiol or tamoxifen (data not shown). This protein is missing the second zinc finger of the DNA binding domain and as such would not be expected to bind to DNA. We found that under conditions in which *in vitro* transcribed/translated wild-type ER- α could bind to an oligonucleotide containing the vitellogenin B1 ERE, d3/4 did not demonstrate any specific DNA binding in gel mobility shift assays (data not shown).

To examine the transcriptional activity of d3/4 variant ER- α , transient transfections using ER negative cell lines were carried out. Under conditions in which transiently transfected wild-

type ER- α was transcriptionally active and able to induce CAT activity in a ligand-dependent fashion, the d3/4 ER- α did not demonstrate any transcriptional activity on its own (data not shown). This is unlikely to be due to low levels of expression of this transgene, since after transfection of 5 μ g of d3/4 vector into MCF10A1 cells we were able to detect a protein corresponding in size to the expected d3/4 protein (Figure 4). To determine if d3/4 ER- α and wild-type ER- α could interact to influence transcription, cotransfections of wild-type and d3/4 ER- α into MDA-MB-231 and MCF10A1 ER negative breast epithelial cell lines were carried out (Figure 5A and B). HEGO transfected alone showed the expected estrogen-dependent activity (Figure 5A and B) while d3/4 alone had no transcriptional activity. However, when increasing amounts of d3/4 ER- α were cotransfected with a constant amount of HEGO into MDA MB 231 cells, d3/4 significantly increased estrogen-dependent activity of wild-type ER- α (ANOVA, $p < 0.0001$). Furthermore, although it did not reach statistical significance there was a trend of increased d3/4 to increase the basal level of activity of the wild-type ER- α . This pattern of activity was also seen when similar experiments were carried out in MCF10A1 cells. A significant increase in the estrogen-dependent activity was seen (ANOVA, $p < 0.05$), with a trend towards increased basal activity.

We next examined the effects of introducing the d3/4 ER- α into the parental T5 cells. Transient transfection of d3/4 into T5 cells was carried out and ER- α transcriptional activity measured. Figure 6 shows the results obtained, and demonstrate that increasing amounts of d3/4 ER- α transiently transfected into T5 cells was associated with a significant increase in CAT activity both in the presence and absence of added estrogen (ANOVA, $p < 0.05$ and $p < 0.0001$, respectively) despite the fact that this variant ER- α does not bind appreciably to ligand *in vitro* nor has transcriptional activity of its own at this concentration.

Discussion.

Numerous studies have identified variant ER- α mRNAs in both normal and neoplastic breast tissue and cell lines (Fuqua *et al.*, 1992; Garcia *et al.*, 1988; Leygue *et al.*, 1996a; Leygue *et al.*, 1996c; McGuire *et al.*, 1992; Murphy & Dotzlaw, 1989b). While still a controversial topic, evidence is emerging to support the existence of ER- α variant proteins, which could correspond to some ER- α variant mRNAs, in some cell lines and tissues *in vivo* (Castles *et al.*, 1993; Fuqua *et al.*, 1992; Marigliante *et al.*, 1991; Monchamont *et al.*, 1991; Montgomery *et al.*, 1993; Pink *et al.*, 1995; Raam *et al.*, 1988; Scott *et al.*, 1991). However, the pathophysiological significance of ER- α variant expression is unclear. Altered expression of some ER- α variant mRNAs was found associated with both breast tumorigenesis and breast cancer progression (Daffada *et al.*, 1995; Fuqua *et al.*, 1992; Leygue *et al.*, 1996b; Leygue *et al.*, 1996c; Murphy *et al.*, 1995). Several studies, using transient transfection analyses, have shown that individual ER- α variant proteins can have both positive and negative effects on wild-type ER- α activity (Castles *et al.*, 1993; Desai *et al.*, 1997; Fuqua *et al.*, 1992; Fuqua *et al.*, 1991; Pink *et al.*, 1995; Pink *et al.*, 1996; Rea & Parker, 1996; Wang & Miksicek, 1991). Conflicting results for some ER- α variants have been obtained (Fuqua *et al.*, 1993; Wang & Miksicek, 1991) which may be due to cell and promoter specific events previously identified for various structure/function domains of the wild-type ER- α (Tora *et al.*, 1989b; Tzukerman *et al.*, 1994). Similarly, overexpression of a single ER- α variant using stable transfection technology has resulted in different results in different laboratories (Fuqua & Wolf, 1995; Rea & Parker, 1996). Moreover, direct correlation of any single ER- α variant with clinical tamoxifen resistance or tamoxifen resistance of breast cancer cells in culture has not been forthcoming. Since most of these comparisons have been performed using individual ER- α variants and do not take into account the entire spectrum of ER- α variants relative to each other, the conclusions remain controversial. Together, the data support the hypothesis that the development of hormone-independence and endocrine resistance in human breast cancer is a multifactorial process and indeed there are many examples where the development of estrogen-independent growth and antiestrogen resistance are dissociable events in breast cancer cell line models (Berg *et al.*, 1990; Brunner *et al.*, 1993; Katzenellenbogen *et al.*, 1987; Welshons & Jordan, 1987). Similar to these and other studies, we have found that the development of estrogen-independent growth in a breast cancer cell line model, through long term growth in estrogen-depleted medium, was not associated with antiestrogen resistance. Although the estrogen-nonresponsive T5-PRF cells have a reduced

sensitivity to the pure antiestrogen ICI 164,384, their growth response to 4-hydroxytamoxifen is identical to parental T5 cells (Coutts *et al.*, 1996). However, when we investigated the relative pattern of expression of ER- α deleted variant mRNA in T5-PRF compared to parental T5 cells, there was a significant difference in the relative expression of a previously described exon 3 plus 4 deleted ER- α variant mRNA (reviewed in Murphy *et al.*, 1997). Although the question of whether this ER- α variant is a cause of estrogen independence or merely an effect of the selection process for estrogen independence requires further study, our data using transient transfection analyses tend to support a possible functional role for the putative 3/4 deleted ER- α protein encoded by the variant mRNA in the phenotype observed in T5-PRF human breast cancer cells. In this study we have shown that T5-PRF cells have significantly increased ligand-independent (basal) ER- α activity (reflected in both ERE-tk-CAT activity and endogenous PR levels). The recombinant d3/4 variant ER- α was able to confer significantly increased ligand-independent (basal) and estrogen-responsive transcriptional activity when expressed in parental T5 cells and showed a trend towards increased basal transcriptional activity when coexpressed with wild-type ER- α in ER- α negative human breast cell lines. While the demonstrated effects of d3/4 on HEGO transcriptional activity in ER- α negative cell lines suggest a putative functional role for this variant ER- α , this effect required equal or higher levels of d3/4 than HEGO. Although our data provide 'proof of principle' that the d3/4 ER- α variant can modulate the transcriptional activity of the wild type ER- α , the relevance of the expression levels of each protein achieved in the reconstituted transient expression system to the endogenous levels of ER- α and d3/4ER- α variant expression in T5-PRF is unclear. Furthermore, differences in the background of transcriptional coactivators and corepressors between naturally ER- α positive and negative cell lines (for example Tzukerman *et al.*, 1994), as well as the presence of other naturally occurring ER- α variants in naturally ER- α positive cell lines are all likely to impact on the final outcome of ER mediated transcriptional activity and underlie the differences seen between the transiently manipulated cells and the naturally occurring T5-PRF phenotype. Moreover, expression of ER- β and/or its variants may influence estrogen action (Giguere *et al.*, 1998). Both T5 and T5-PRF cells express low levels of ER- β mRNA determined by reverse transcription polymerase chain reaction analysis (Dotzlaw *et al.*, 1997), unpublished data), however, the functional significance of the levels remains unknown. Nonetheless, we saw a significant effect on ER- α ligand-independent transcription in T5 cells at levels of co-transfected d3/4 that likely would not be higher than the endogenous ER- α in these cells, but the extrapolation

of these data to the relative expression of wild type ER- α and d3/4 ER- α variant in T5-PRF cells is presently unknown. It is of significance that we can reproduce an effect of d3/4 variant in the parental T5 cells, which would likely contain a more representative background of ER- α accessory proteins (i.e. coactivators and/or corepressors) as well as other variant forms of ER- α which would all contribute to the final ER mediated biological response. As well, our data do not exclude the possibility that other alterations have occurred in T5-PRF cells which, in combination with an altered ER- α variant, may contribute to the estrogen-independent phenotype of T5-PRF cells (Coutts & Murphy, 1998).

It has previously been shown that breast cancer cells can adapt to low levels of estrogen by enhancing their sensitivity to estrogen (Masamura *et al.*, 1995). Estrogen-deprivation of MCF-7 human breast cancer cells resulted in estrogen hypersensitivity and maximal growth was achieved with an estrogen concentration 4-5 orders of magnitude lower than wild-type cells. These researchers also found that the concentration of ICI needed to inhibit growth of these cells was ~6 orders of magnitude lower than wild-type cells, supporting the hypothesis in this model, that increased sensitivity to ER ligands had occurred. While supersensitivity to estrogen in T5-PRF cells cannot be entirely ruled out, we have previously shown that in contrast to the data of Masamura *et al.*, T5-PRF cells are less sensitive to growth inhibition by ICI, suggesting that in this model other mechanisms are likely involved.

Our data do not address the mechanism by which d3/4 enhances ER transcriptional activity, but several possibilities exist. The ER- α contains at least two separate regions that are required for optimal transcriptional activation (Tora *et al.*, 1989b; Tzukerman *et al.*, 1990). The amino-terminal region contains promoter and cell-type specific ligand-independent transcriptional activity (AF1) and the second, AF2, is located in the ligand-binding carboxyl-terminus of the receptor. Exon 3/4 deleted ER- α containing an intact AF2 or AF1 domain could interfere with, or sequester, an ER- α repressor protein resulting in increased ER- α transcriptional activity in the absence of ligand (Lee *et al.*, 1996). This variant may also retain the ability to interact with other ER- α regulatory proteins such as coactivators or components of the basal transcription machinery.

ER- α also contains two domains involved in dimerization (Kumar & Chambon, 1988; Schwabe *et al.*, 1993). A weak dimerization interface is present in the DNA-binding domain (DBD) and a strong interface is located in the C-terminal ligand-binding domain (White *et al.*, 1991). d3/4

containing an intact C-terminal dimerization domain, may form heterodimers with wild-type ER- α that have altered transcriptional regulatory properties through differing protein-protein interactions.

The crystal structure of the ER- α hormone binding domain has recently been elucidated (Brzozowski *et al.*, 1997). Based on this structure, the d3/4 protein would contain many of the regions essential for transactivation, including the predominant helix 12 (encompassing amino acids 539-547). However, since d3/4 alone has no transcriptional activity (at least on a classical ERE regulated promoter) the structure must be sufficiently altered to prevent activity, or AF2 can only be activated in a ligand-dependent manner but d3/4 cannot bind ligand. Helix 12 in AF2 is believed to be the main region involved in coactivator recruitment and it may be possible that d3/4, following heterodimerization with ER- α enhances recruitment of coactivators to the basal transcription complex and this enhances ER- α activity.

We have found that the d3/4 ER- α caused increased ligand-independent wild-type ER- α activity and also enhances the ligand-induced ER- α transcriptional activity, despite the fact that on its own this variant is not transcriptionally active on a classical ERE promoter, nor does it bind ligand *in vitro* to any significant degree. Studies have demonstrated that the ability of steroid hormone receptors to modulate transcription does not necessarily require that the receptors bind DNA. PRc, an N-terminally truncated PR isoform lacking the first zinc-finger of the DBD, has no transcriptional activity of its own but has been shown to enhance progestin-induced transcriptional activity (Wei *et al.*, 1996). The DBD of the ER- α does not appear to be necessary for raloxifene activation of the TGF β 3 gene (Yang *et al.*, 1996) and ER- α can activate transcription from AP-1 dependent promoters through a DNA binding-independent pathway (Webb *et al.*, 1995). Sp1 and ER- α directly interact to enhance estrogen-induced transactivation of the Sp1-dependent Hsp27 gene promoter and the DBD of the ER- α is not required (Porter *et al.*, 1997).

Recent research has demonstrated that the ER- α can be activated in a ligand-independent fashion (Ignar-Trowbridge *et al.*, 1993). Studies have shown that several growth factors such as epidermal growth factor (EGF), transforming growth factor alpha (TGF α) and insulin-like growth factor (IGF-1) were able to activate the ER- α in the absence of ligand. The ability to activate the ER- α in the absence of estrogen could confer a growth advantage to breast cancer cells and aid in the development of a hormone-independent phenotype. The presence of alternate forms of ER- α capable of interacting with wild-type ER- α to increase ligand-independent activity could also confer a potential growth advantage to breast cancer cells. A recent study has shown that

constitutively active, ligand-independent ER- α mutants undergo conformational changes and interactions with coactivators that mimic changes in ER- α that are usually regulated by ligand (Lazennec *et al.*, 1997). Recently, researchers have shown that TR- β 2 is a ligand-independent activator of the gene encoding thyrotropin-releasing hormone (TRH) and have mapped a region in the N-terminus of the receptor responsible for this activity (Langlois *et al.*, 1997). These researchers suggest that the mechanism of ligand-independent activation involves direct interaction of the TR- β 2 amino terminus with either transcriptional cofactors or the basal transcription machinery itself.

An increased relative expression of variant ER- α proteins containing intact AF domains, could result in increased interactions with the ER- α and/or other proteins involved in ER- α transcriptional activity. This could be a potential mechanism for estrogen-independent growth associated with the presence of one or more variant ER species and could explain the increased ER- α activity we have seen with the d3/4 ER- α .

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FIGURE LEGENDS

Figure 1. ER transcriptional activity. **A.** T5 and T5-PRF cells were transfected and CAT assays performed as described in Methods. Results are expressed as fold CAT activity compared to T5 basal (arbitrarily set at 1.0). * $p < 0.05$, Student's t-test (compared to T5 basal). Results represent mean \pm SEM, $n=7$. **B.** T5-PRF cells were transfected and CAT assays performed as described in Methods. Results represent percent CAT activity as compared to T5-PRF basal set at 100%, mean \pm range, $n=2$.

Figure 2. Progesterone receptor levels. PR levels were determined by whole cell binding as described in Methods. PR levels are expressed as fmol PR/ 10^6 cells and results represent mean \pm SEM, $n=3$. ** $p < 0.01$, Student's t-test.

Figure 3. Identification of exon 3/4 deleted variant ER- α mRNA. **A.** Long-range ER- α RT-PCR. Total RNA was extracted from T5 and T5-PRF cells, reverse transcribed and PCR amplified using 1/8U and 1/8L primers. Labelled PCR products were separated on 3.5% acrylamide-urea gels and visualized using autoradiography. WT-ER = wild type ER- α , D7-ER = exon 7 deleted ER- α , D4-ER = exon 4 deleted ER- α and D3/4-ER = exon 3 and 4 deleted ER- α , based on size compared to labelled markers (not shown). **B.** Sequence of d3/4 ER- α cDNA. The 928bp PCR product was excised from a gel and subcloned (in triplicate) and three colonies from each independent subcloning were sequenced to confirm the identity of d3/4 cDNA.

Figure 4. *In vivo* expression of d3/4 protein. MCF10A1 human breast epithelial cells were transfected with the appropriate expression vectors, cells were lysed in 8M urea and 10 μ g protein run on a 10% SDS-acrylamide gel. Gels were transferred to nitrocellulose and Western blotting performed using ER- α Ab H226. Lane 1: control cells transfected with 5 μ g of vector (pSG5) alone; lane 2: cells transfected with 5 μ g HEGO (WT-ER- α) expression vector; lane 3: cells transfected with 5 μ g d3/4 expression vector; lane 4: *In vitro* transcribed/translated WT-ER- α (1 μ l); lane 5: *In vitro* transcribed/translated d3/4 ER- α (2 μ l).

Figure 5. Activity of d3/4 in ER- α negative cells. A. MDA-MB-231 cells were transfected with 5 μ g ERE-tk-CAT, 1 μ g pCH110, 0.5pmol HEGO \pm 0.5-2pmol d3/4 \pm vector DNA to give a total of 17 μ g DNA/dish. Cells were treated with 10nM estradiol (E2) for 24h or vehicle alone as control. Results are expressed as fold CAT activity compared to basal HEGO activity arbitrarily set as 1. Histograms represent mean + SEM, n=6-7. ***e = p < 0.0001 by ANOVA results compared to estradiol treated HEGO alone. B. MCF10A1 cells were similarly transfected. Histograms represent mean + SEM, n=4. *e = p < 0.05, ANOVA, result compared to estradiol treated HEGO alone.

Figure 6. Transient transfection of d3/4 expression vector into T5 cells. Cells were grown in PRF-DMEM as described in Methods and transfected with 5 μ g ERE-tk-CAT expression vector, 5 μ g pCH110 along with the appropriate amount of d3/4 expression vector. Cells were treated with vehicle or 10nM estradiol (E2) for 24h, harvested and CAT assays performed. Results represent mean + SEM, n=3-5, ***b = p < 0.0001, ANOVA, result compared to control basal ERE-tk-CAT activity. *e = p < 0.05, ANOVA, result compared to control estradiol treated ERE-tk-CAT activity.

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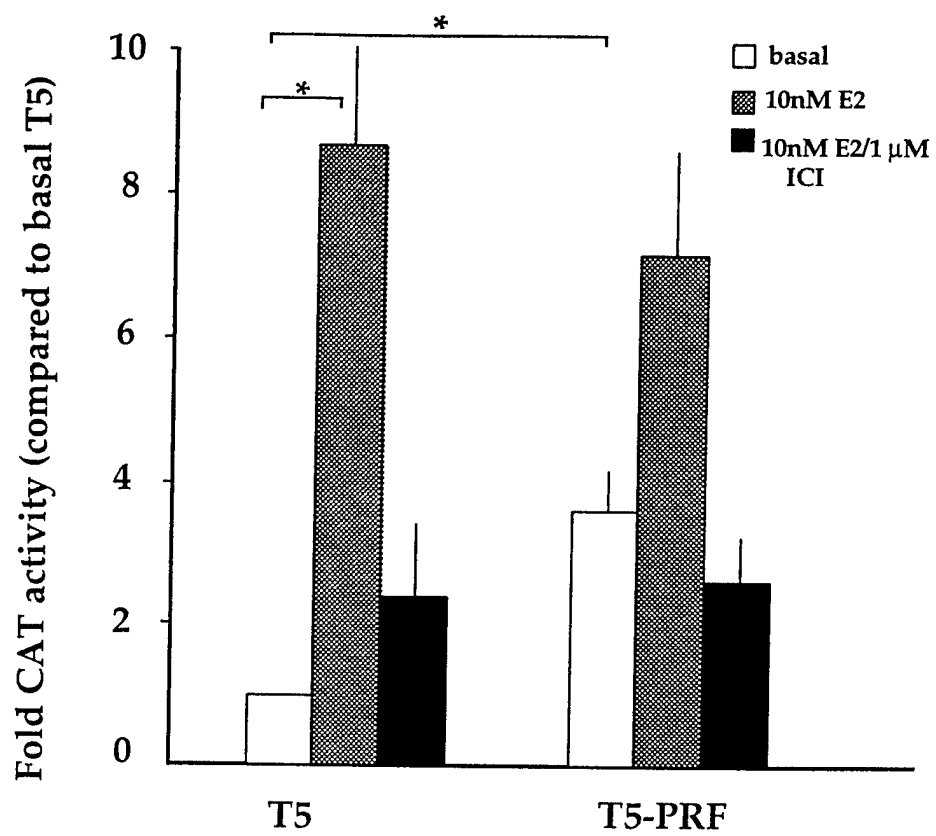
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Figure 1

A



B

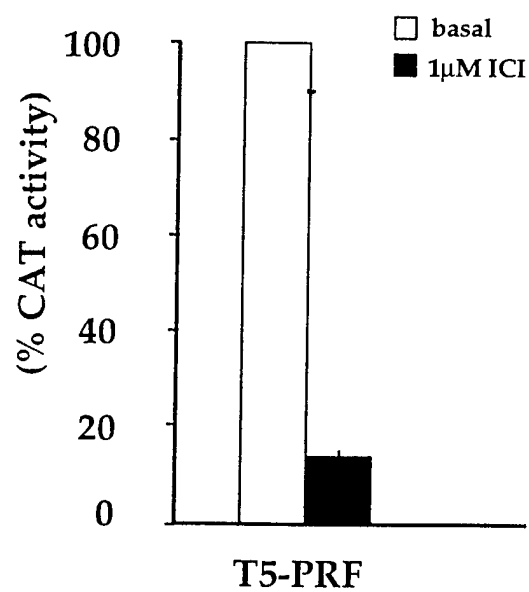


Figure 2

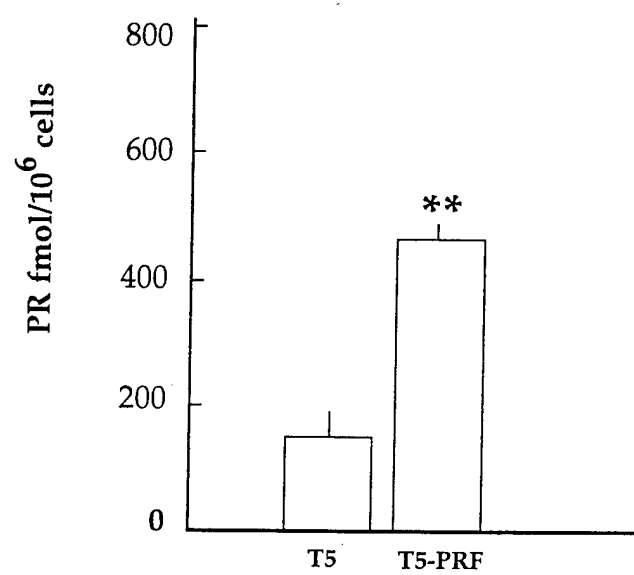
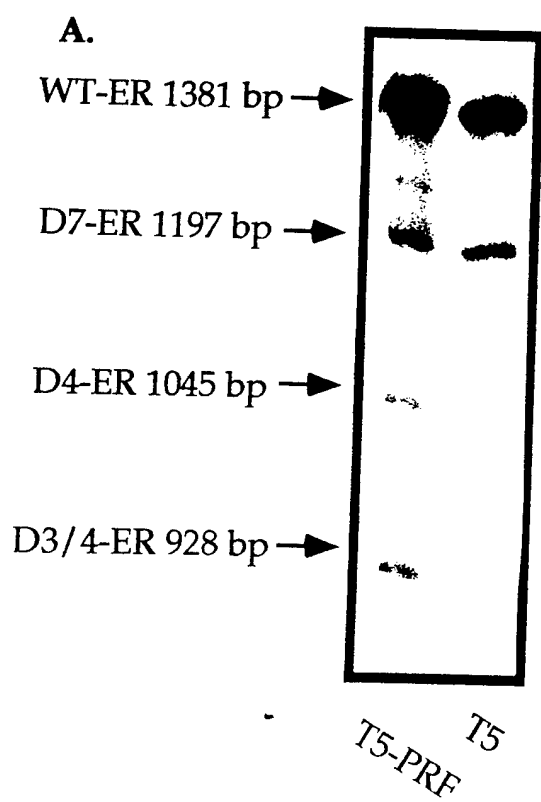


Figure 3.



B.

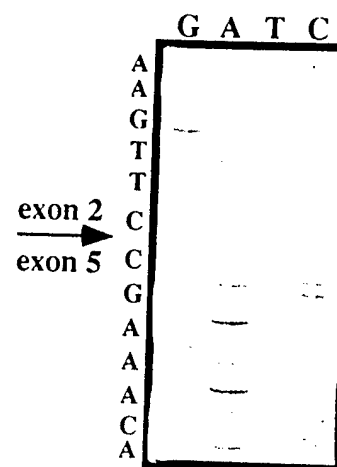


Figure 4.

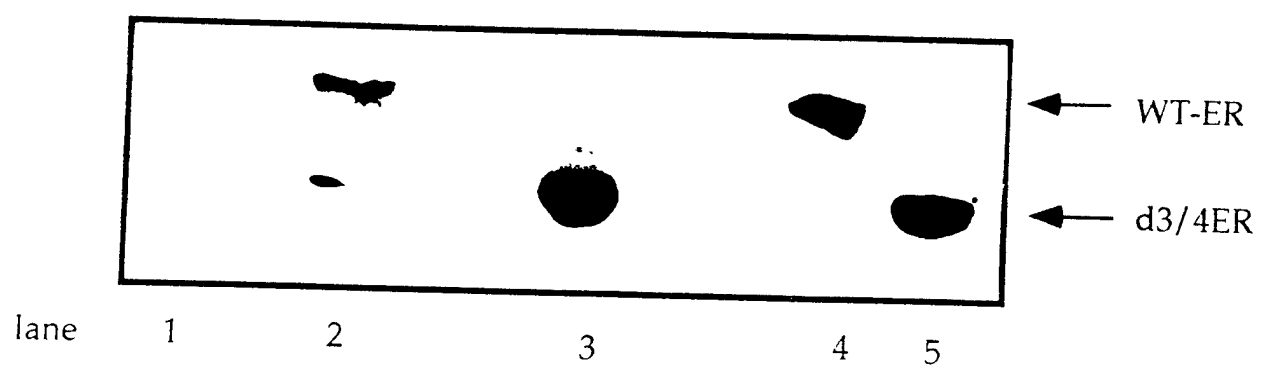
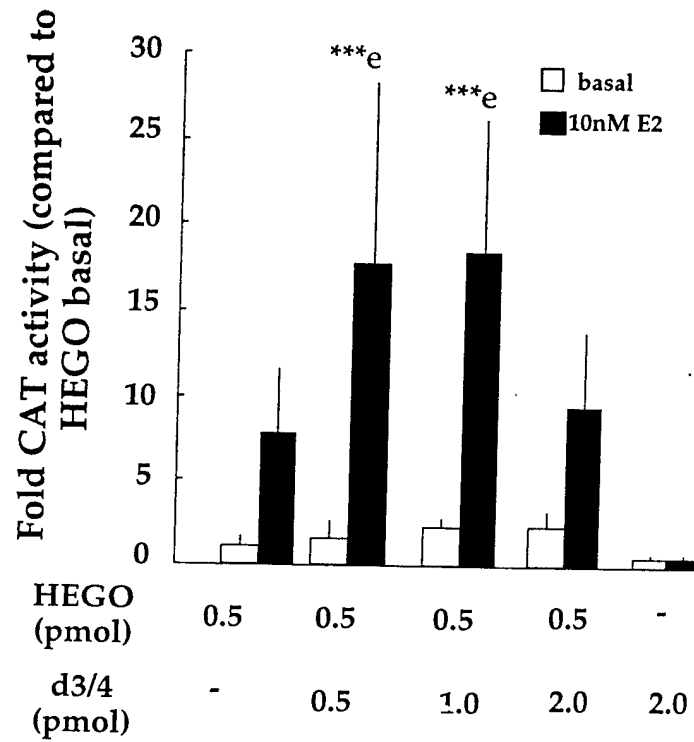


Figure 5.

A



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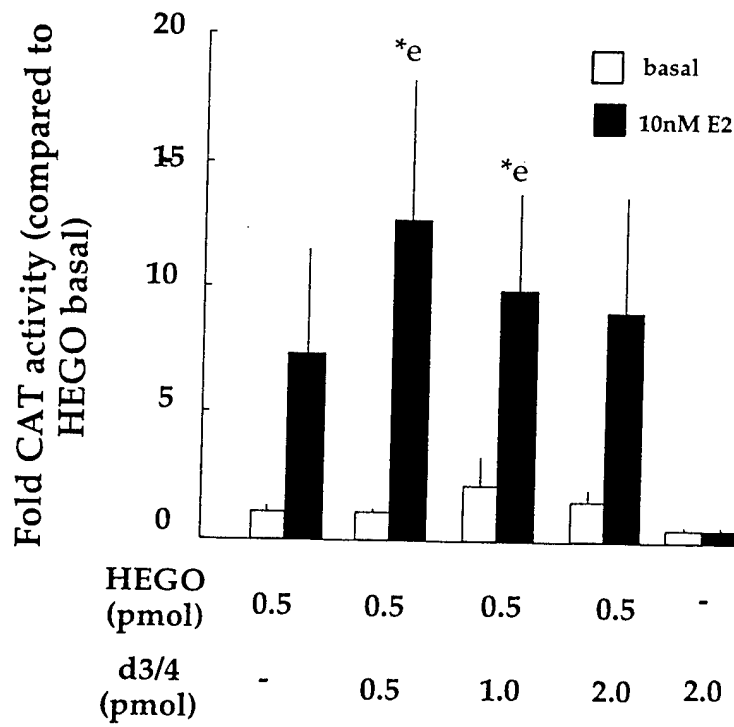
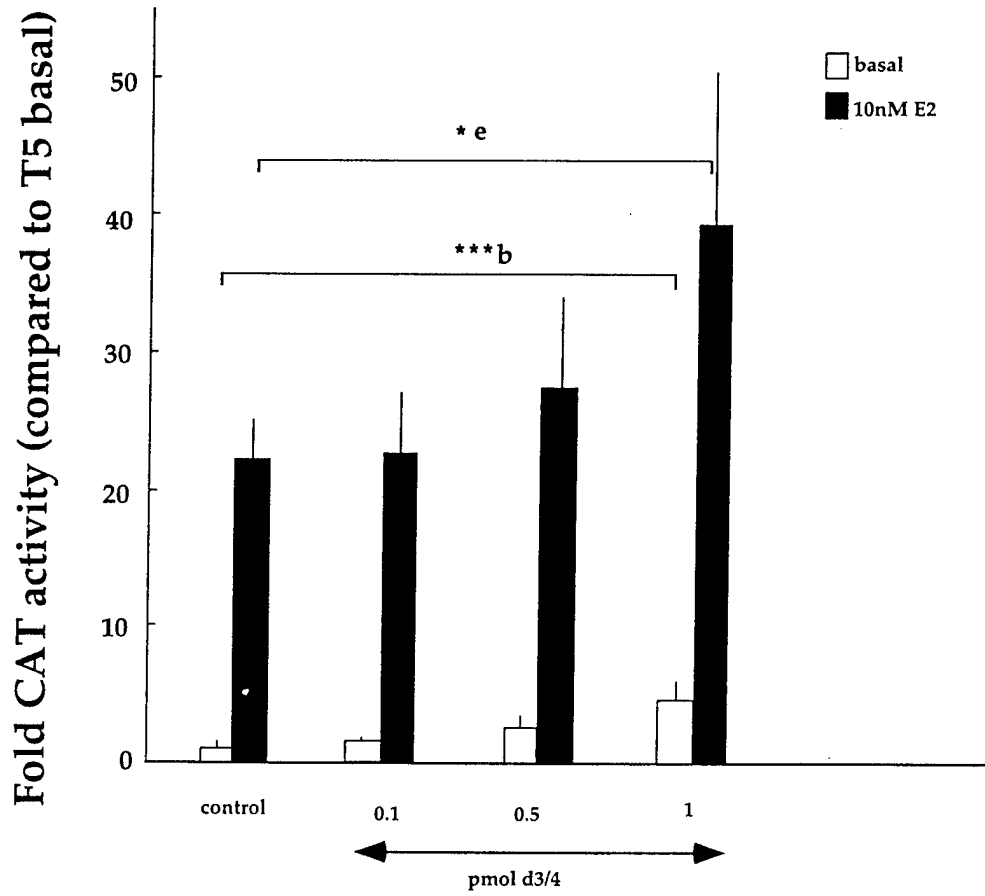


Figure 6.



APPENDIX 7

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Identification of novel exon-deleted progesterone receptor variant mRNAs
in human breast tissue.

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Identification of Novel Exon-Deleted Progesterone Receptor Variant mRNAs in Human Breast Tissue

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Using an approach based on the co-amplification of wild-type and exon deleted progesterone receptor (PR) variant cDNAs, we identified exon-deleted PR variant mRNAs in both normal and neoplastic human breast tissues. Several naturally occurring variants, whose sequences revealed precise whole exon deletions, may encode putative PR-like proteins which lack some functional domains of the wild-type PR molecule. We suggest that these PR variant proteins could have a pathophysiological role in progestin action, as suggested for estrogen receptor variant proteins. © 1996 Academic Press, Inc.

The progesterone receptor (PR), which belongs to the superfamily of ligand-activated nuclear transcription factors (1), is essential for progestin action in target tissues such as the endometrium and mammary gland. PR is an important prognostic marker in breast cancer as well as a marker of responsiveness to endocrine therapies (2). Its presence in estrogen receptor (ER) positive breast tumors generally indicates a high likelihood of responsiveness to endocrine agents (3-4). In contrast, PR absence is often associated with failure to respond to these agents (5). Like all other members of the steroid/thyroid/retinoic acid receptor superfamily (6), PR is divided into structural and functional domains (A-E, Figure 1). Upon binding of ligand, PR dimerizes, undergoes phosphorylation and binds to specific sequences (PRE) located in the 5' flanking region of PR-responsive genes (7). Further rounds of phosphorylation depending on DNA binding are also involved in transcriptional activation of the PR (8). Depending on the ligand, the isoforms involved (PR-A or PR-B), the target cell type, and the targeted gene, PR-activation will result in increased or decreased gene transcription (8-11).

Two functionally different PR isoforms, PR-A and PR-B (769 and 933 amino acids, respectively), have been previously identified in both normal and neoplastic human tissues (12). These two PR isoforms differ only in that PR-A lacks the NH₂-terminal 164 amino acids of PR-B. PR-A and PR-B are translated from two distinct groups of mRNAs transcribed from the same gene under the control of two different promoters (Figure 1, 13). A third PR isoform (PR-C), that would be encoded by mRNAs lacking the translational start sites of PR-B and PR-A mRNAs but whose exact amino acid composition has not yet been established, has also been described (14). Beside the Mr 90,000 PR-A, Mr 120,000 PR-B and Mr 60,000 PR-C proteins, several other PR-related proteins have been observed by Western blot in human breast tumors (15) and in T47D breast cancer cells (14). Characterization of these PR-related proteins and their possible significance in progesterone action still remain unclear. Furthermore, several PR-related mRNAs, ranging in size from 11.4 kb to 2.5 kb, as determined by Northern blot analysis, have been observed in both normal and neoplastic human tissues (16, 17). The origin of all these mRNAs remains unknown, although alternative promoter usage, alternative polyadenylation site selection and absence of

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Abbreviations: PR, progesterone receptor; ER, estrogen receptor; WT, wild-type; PCR, polymerase chain reaction.

splicing have been suggested (17, 18). Although several of these mRNAs could encode PR-A and PR-B, other transcripts could not encode either of these isoforms (17, 18). The significance of the diversity of PR transcripts is therefore unclear. By analogy to the human ER and other members of the steroid receptor superfamily, we hypothesized that the diversity in PR-related transcripts could partly result from differential splicing. Several exon-deleted or truncated ER variant mRNAs have been observed in both normal and neoplastic tissues (19-24). The altered expression of some of these ER variant mRNAs and possibly the putative proteins encoded by these ER variant mRNAs, that lack some of the wild-type (WT) ER functional domains, has been suggested to be involved in the hormone independent phenotype of some breast tumors as well as in breast tumorigenesis (5, 24-28). It was therefore of interest to determine if similar exon-deleted PR variant mRNAs could also be observed in human breast tissues.

MATERIALS AND METHODS

Human breast tissues. Human breast tumor specimens (24 cases) and normal breast tissues obtained from reduction mammoplasty surgical specimens (10 cases), were collected at the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The presence of normal ducts and lobules was confirmed in all normal tissue specimens, as well as the absence of any atypical lesion. The twenty four primary invasive ductal breast carcinomas were associated with ER levels ranging from 0.5 to 386 fmol/mg protein, as determined by ligand binding assay. Within this group, 11 tumors were progesterone receptor positive (PR>15 fmol/mg protein), 12 were borderline positive (<15 fmol/mg protein) and 1 was PR negative (PR=0 fmol/mg protein), as also determined by ligand binding assay. The breast cancer cell line T-47D-5 was kindly provided by Dr. RL Sutherland (Garvan Institute for Medical Research, Sydney, Australia). These cells were previously shown to contain a high level of PR mRNA (29) and have therefore been used as positive controls. Total RNA was extracted and reverse transcribed in a final volume of 15 μ l as previously described (24).

Polymerase chain reaction (PCR) and identification of PCR-products. The primers used consisted of PRU2 primer (5'-CCAGCCAGAGCCCACAATACA-3'; sense; located in PR exon 2; 2395-2415) and PRL2 primer (5'-GCAGCAATAACTCAGACATC-3'; antisense; located in PR exon 8; 3487-3467). The nucleotide positions given correspond to published sequences of the human PR cDNA (13). PCR amplifications were performed and PCR products analyzed as previously described (24). Briefly, 1 μ l of reverse transcription mixture was amplified in a final volume of 10 μ l, in the presence of 10 nM [α -³²P] dCTP, 4 ng/ μ l of each primer and 1 unit of Taq DNA polymerase. Each PCR consisted of 40 cycles (1 minute at 60°C, 2 minutes at 72°C and 1 minute at 94°C). PCR products were then separated on 4% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and autoradiographed. In order to control for errors in input of cDNA used in PCR reactions, amplification of the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as previously described (24). PCR products were subcloned and sequenced as previously described (24). Predicted molecule mass and isoelectric point of the putative proteins encoded by the PR variant mRNAs identified in this study were obtained using MacVector™ 4.1.4 software (Kodak Scientific Imaging System, New Haven, CT).

RESULTS

Co-amplification of wild-type and exon-deleted PR mRNAs in breast samples. We used an approach adapted from a recently developed strategy used to study the prevalence of ER variant mRNAs within tumor samples (30). This approach is depicted in Figure 1. cDNAs corresponding to exon-deleted PR variants could be amplified together with the WT-PR mRNA using primers annealing with exon 2 (PRU2) and exon 8 (PRL2) sequences. In order to amplify variant mRNAs possibly related to both PR-A- and PR-B mRNAs, we have confined our approach to the region within exon 2 and exon 8, shared by these two mRNAs. Ten normal breast tissue samples obtained from reduction mammoplasties, and 24 breast tumor samples with a wide range of ER and PR levels, were studied. Total RNA was extracted from each sample, reverse transcribed and PCR performed in the presence of radiolabelled nucleotide. Figure 2 shows typical results obtained. Several different PCR products were observed in both normal and tumor samples. Three bands, that migrated with the apparent sizes of 1093 bp, 966 bp and 794 bp were observed reproducibly (i.e. in at least two independent experiments) in most normal and neoplastic breast tissue samples, although the relative abundance of the bands seemed to differ amongst samples. These same bands were also observed in the PR positive T47D-5 human breast cancer cell line. Following subcloning

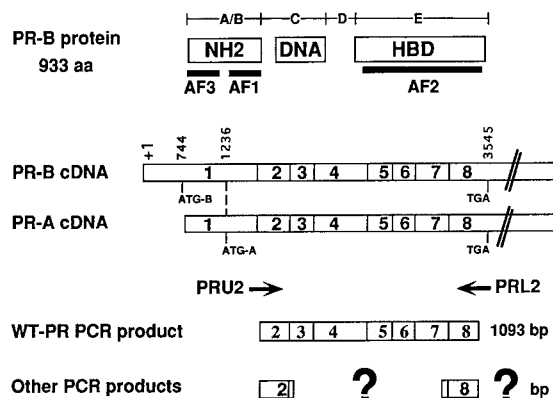


FIG. 1. Schematic representation of PR-B protein, PR-A and PR-B cDNAs, and primers allowing co-amplification of exon-deleted PR variant cDNAs. PR cDNAs contain 8 different exons coding for a protein divided into structural and functional domains (A-E). The NH₂-terminal region A/B of the receptor contains two transactivation functions (AF3 and AF1). The DNA-binding domain of the receptor is located in the C region. Region D corresponds to the hinge domain of the protein and region E is involved in hormone binding and contains another transactivation domain (AF2). ATG-B and ATG-A are the translational start sites of PR-B and PR-A, respectively. PRU2 and PRL2 primers allow amplification of a 1093 bp fragment corresponding to WT-PR mRNAs. Co-amplification of all possible exon-deleted variants that contain exon 2 and exon 8 sequences can occur.

and sequencing, these bands were shown to correspond to the WT-PR mRNA, exon-6 deleted and exon-4 deleted PR variant mRNAs, respectively. Sequences of these variants showed a perfect junction between exons (31) surrounding the deletion area as shown in Figure 3. Two other bands, that migrated with the apparent size of 845 bp and 817 bp were also detected in some breast tumor samples, but at an apparent lower frequency than the bands corresponding to WT, exon 4-deleted and exon 6-deleted PR mRNAs. Sequencing of these bands identified a PR variant transcript containing a deletion of both exon 3 and 6, as well as a transcript containing a deletion of both exon 5 and 6 (data not shown). Some other PCR products, which were not reproducibly observed or whose size did not correspond to any putative exon-deleted PR variant mRNA have not yet been characterized. Differences between samples in PR wild-type and variant mRNAs signals are unlikely due to variable input of cDNA, since similar signals were obtained in all samples after amplification of the house-keeping GAPDH cDNA (data not shown). Generally, there was good agreement between ligand binding assay and the wild-type PR reverse transcription (RT) PCR product obtained. Specificity of the RT-PCR approach is demonstrated by the lack of signal in a PR negative tumor (T2), as measured by ligand binding assay. Table 1 summarizes the characteristics (size, predicted molecular mass, predicted isoelectric point) of the putative proteins encoded by the PR variants identified in this study. Because both PR-A and PR-B type variant mRNAs will be identified using our approach, both types of putative protein are presented (we have not analyzed our data with respect to the putative PR-C isoform). Intact functional domains that remain in the resulting protein are indicated for each PR-variant. It should be emphasized that to date, only RT-PCR bands corresponding to the exon 4-deleted and exon 6-deleted variant mRNAs in addition to the wild-type PR mRNA were reproducibly detected in normal breast tissue. Our data suggest that the exon 6-deleted transcript is more frequently detected in neoplastic breast tissues compared to the normal breast tissues examined in this study.

DISCUSSION

We have identified for the first time several exon-deleted PR variant mRNAs present in both normal and neoplastic breast tissues. As previously observed with exon-deleted ER

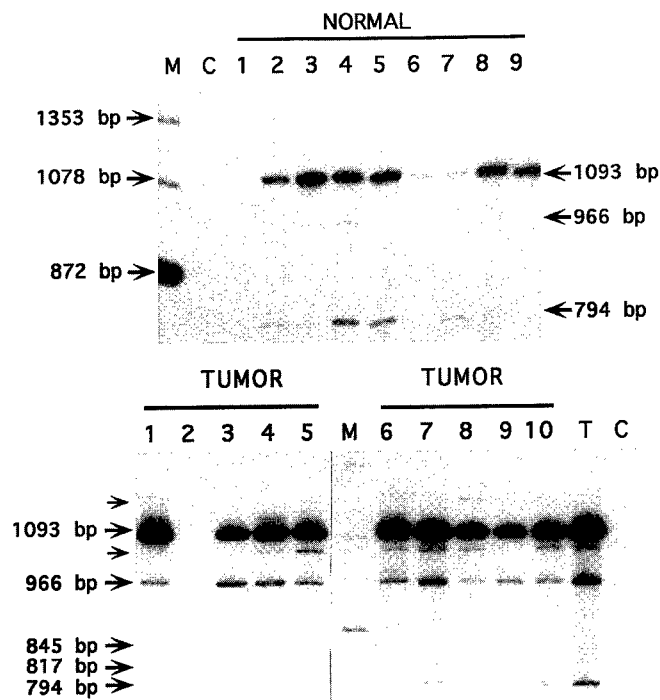


FIG. 2. Co-amplification of WT-ER and deleted variant mRNAs in human breast samples. Total RNA extracted from normal (Normal 1-9) and tumorous (Tumor 1-5 and 6-10) breast tissue samples or from T47D-5 breast cancer cells (T) was reverse transcribed and PCR amplified as described in the "Material and Methods" section using PRU2 and PRL2 primers. Radioactive PCR products were separated on a 4% acrylamide gel and visualized by autoradiography. Bands that migrated at 1093 bp, 966 bp, 845 bp, 817 bp and 794 bp were identified as corresponding to WT-PR mRNA and variant mRNAs deleted in exon 6, doubly deleted in exon 3-6, doubly deleted in exon 5-6 and deleted in exon 4, respectively. PCR products indicated by small arrows have not yet been characterized. M: Molecular weight marker (ϕ x174 HaeIII digest, Gibco BRL, Grand Island, NY). C, no cDNA added during the PCR reaction.

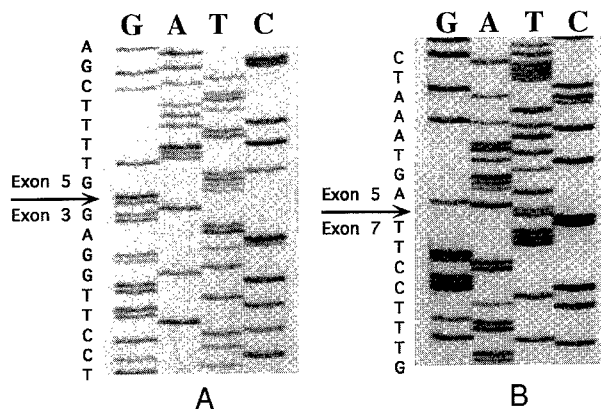


FIG. 3. PR-variant mRNA sequences. PCR products were subcloned and sequenced as described. A: exon 4-deleted variant sequence, B: exon 6-deleted variant sequence.

TABLE 1
PR Variant mRNAs Identified within Human Breast Tissues and Putative Encoded Proteins

	PR-B mRNA	PR-A mRNA	Functional domains	Samples
Wild-type	933 aa <i>Mr</i> 99,035 pI 6.07	769 aa <i>Mr</i> 82,350 pI 8.50	A, B, C, D, E	N, T
Exon 4-deleted variant	831 aa <i>Mr</i> 87,788 pI 5.62	667 aa <i>Mr</i> 71,103 pI 6.97	A, B, E in frame	N, T
Exon 6-deleted variant	797 aa <i>Mr</i> 83,017 pI 5.55	633 aa <i>Mr</i> 66,332 pI 7.42	A, B, C, D truncated, 12 new aa	N, T
Exon 5-6-deleted variant	841 aa <i>Mr</i> 88,068 pI 6.06	677 aa <i>Mr</i> 71,383 pI 8.71	A, B, C, D in frame	T
Exon 3-6-deleted variant	758 aa <i>Mr</i> 78,671 pI 5.03	594 aa <i>Mr</i> 61,985 pI 5.80	A, B, D truncated, 12 new aa	T

Note. For each PR-A or PR-B variant mRNA, the size (in amino acids, aa) of the putative encoded protein, its predicted molecular weight (*Mr*, given in daltons) and its predicted isoelectric point (pI) are given. Functional domains that remain intact are indicated (A-E) as well as the amino acid composition change. Detection in normal (N) or in tumor (T) breast samples is also specified.

variant mRNAs, sequencing these variant mRNAs revealed a perfect junction between exons surrounding the deletion area. This suggests that these naturally occurring variants are generated by alternative splicing of WT-PR primary transcripts. Whether or not all groups of mRNAs (PR-A, PR-B, PR-C) are alternatively spliced remains to be determined. However, any of these variant species, if translated, would encode PR-like proteins which lack some functional domains of the WT-PR proteins. The resulting shorter proteins could contribute to the population of PR-related proteins observed in human breast tumor samples by Graham et al. (15), as suggested by these authors. For example, the putative protein encoded by the exon 4-deleted PR-A variant mRNA is expected to migrate at a apparent molecular mass of 71,103 daltons and could correspond to the PR-related protein (78,000 daltons) observed in 25.7% of the tumors analyzed by Graham et al. (15).

Several different sized PR mRNAs species have previously been described in human and chicken target tissues. Generation of these transcripts is thought to involve several mechanisms: different promoter usage, alternative polyadenylation site selection and a splicing variant have been identified (17, 18). The splicing variant, identified in chicken oviduct (18), seems to consist of a failure to splice the second intron followed by polyadenylation site selection within this intron. The resulting transcript therefore consists of exons 1 and 2 with some intron 2 sequences followed by a polyadenylation signal and a poly-A tail. However, no previous studies have identified complete exon-deletions in PR transcripts. Such deletions would have escaped detection by previous studies using Northern blot and differential hybridization analysis.

By analogy with ER variant mRNAs (20, 21, 32) it is reasonable to hypothesize that the putative encoded PR variant proteins, with structural and functional alterations, may modify WT-PR functions. The presence of PR variant mRNAs in normal tissue therefore suggests that the PR signalling pathway involves more protagonists than PR-A, PR-B or PR-C. Moreover, we showed that the detection of a particular variant using this kind of approach depended on the initial representation of this mRNA within the related-mRNAs population (30). Our results suggest that the differences in PR-variant mRNAs detection between samples may therefore

reflect different relative proportions of these variants within samples. Whether or not differential PR variant mRNA expression is associated with a pathophysiological role in progestin action is under investigation.

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APPENDIX 8

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Altered expression of exon 6 deleted progesterone receptor variant mRNA
between normal human breast and breast tumor tissues.

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Altered expression of exon 6 deleted progesterone receptor variant mRNA between normal human breast and breast tumour tissues

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Summary The progesterone receptor (PR) is an important prognostic marker in breast cancer as well as a marker of responsiveness to endocrine therapies. The presence of several exon-deleted PR variant mRNAs in both normal and neoplastic breast samples has recently been reported. Amongst them, a variant mRNA deleted in exon 6 (D6-PR mRNA) that if translated would encode a truncated PR-like protein missing the hormone binding domain and one of the transactivating domains of the wild-type PR protein. In order to determine whether changes in D6-PR variant expression could occur during tumorigenesis, its expression was investigated by reverse transcription and polymerase chain reaction in ten normal reduction mammoplasty samples, nine breast tumours with high PR levels (> 100 fmol mg⁻¹ protein) and eight breast tumours with low PR levels (< 15 fmol mg⁻¹ protein), as determined by ligand binding assay. The relative expression of D6-PR to wild-type PR mRNA was lower ($P < 0.01$) in normal than in all tumour breast samples. Moreover, a trend to lower ($P < 0.1$) relative D6-PR expression was observed in high PR tumours, compared to low PR tumours. These data suggest that increased expression of D6-PR occurs during tumorigenesis.

Keywords: progesterone receptor; breast cancer; tumour progression; variant mRNA

The progesterone receptor (PR) is an important prognostic marker in breast cancer (Horwitz et al, 1975). Oestrogen receptor (ER)-positive breast tumours that also contain PR are considered 'good prognosis' tumours and are likely to respond to endocrine therapies (Horwitz et al, 1978). In contrast, absence of PR often characterizes 'poor prognosis' tumours (ER + PR-) and resistance to endocrine therapy (McGuire et al, 1991).

Similar to other members of the steroid receptor superfamily, PR is divided into structural domains (A-E) (Figure 1), the functions of which are widely documented (Tsai and O'malley, 1994). Two different PR isoforms, PR-A and PR-B, that are encoded by mRNAs transcribed from the same gene under the control of two different promoters (Kastner et al, 1990), have been identified in both normal and neoplastic tissues (Figure 1). The presence of several exon-deleted PR variant mRNAs in both normal and neoplastic breast samples has recently been reported (Leygue et al, 1996a; Richer et al, 1998; Yeates et al, 1998). Amongst them was a variant mRNA deleted in exon 6 (D6-PR mRNA) that, if translated, would encode a PR-A-like and/or a PR-B-like protein containing a truncated E domain. This variant, missing the hormone binding domain and one of the transactivating domains (AF-2) of the wild-type (WT) PR protein, has been shown to act in-vitro as a dominant-negative transcriptional inhibitor of PR-A and PR-B isoforms (Richer et al, 1998). Because expression of analogous exon-deleted or truncated variants has been associated with tumour progression in the case of ER variant

mRNAs (for review see Murphy et al, 1997), it was of interest to determine whether D6-PR variant expression was also modified during tumorigenesis. In this study, we investigated D6-PR variant expression by reverse transcription polymerase chain reaction (RT-PCR) in ten normal reduction mammoplasties samples, nine breast tumours with high PR levels (considered 'good prognostic' tumours) and eight breast tumours with low PR levels (considered 'poor prognosis' tumours).

MATERIALS AND METHODS

Human breast tissues

Human breast specimens (27 cases) were from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The processing of specimens collected in the Manitoba Breast Tumor Bank has already been described (Hiller et al, 1996). Briefly, each specimen was rapidly frozen as soon as possible after surgical removal. A portion of the frozen tissue block was processed to create a paraffin-embedded tissue block matched and orientated relative to the remaining frozen block. These paraffin blocks provide high quality histologic sections, which are used for pathologic interpretation and assessment and are mirror images of the frozen sections used for RNA extractions. For each case, tumour and normal tissues were histologically characterized by observation of paraffin sections. The presence of normal ducts and lobules as well as the absence of any proliferative lesion were confirmed in all ten normal reduction mammoplasties specimens. The 17 primary invasive ductal breast carcinomas were associated with high ER levels ranging from 105 to 386 fmol mg⁻¹ protein (mean = 190.7 fmol mg⁻¹ protein, standard deviation (s.d.) = 58.49), as determined by ligand binding assay. Within this group,

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nine tumours had a high PR level (PR > 100 fmol mg⁻¹ protein, mean = 156.4 fmol mg⁻¹ protein, s.d. = 28.4) and eight had a low PR level (PR < 15 fmol mg⁻¹ protein, mean = 8.6 fmol mg⁻¹ protein, s.d. = 4.6), as determined by ligand binding assay. The ages of patients associated with the tumour samples ranged from 37 to 91 (mean: 70 years old, s.d.: 14.4 years). For reduction mammoplasties, women were younger, with ages ranging from 19 to 41 years old (mean: 31.3 years old, s.d.: 8.3 years). Total RNA was extracted from frozen tissue and reverse transcribed in a final volume of 15 µl as previously described (Leygue et al, 1996b).

PCR and identification of PCR products

The primers used consisted of D6U primer (5'-CTCT-CATTCACTATTCTTGG-3'; sense; located in PR exon 5; 2989-23008) and D6L primer (5'-TGGGTTTGACTTCGTAGC-3'; antisense; located in PR exon 7; 3262-3245). The nucleotide positions correspond to published sequences of the human PR cDNA (Kastner et al, 1990). PCR amplifications were performed and PCR products analysed as previously described, with minor modifications (Leygue et al, 1996b). Briefly, 1 µl of reverse transcription mixture was amplified in a final volume of 10 µl, in the presence of 10 nmol⁻¹ [α -³²P] dCTP, 4 ng µl⁻¹ of each primer and 0.3 unit of *Taq* DNA polymerase. Each PCR consisted of 40 cycles (30 s at 60°C and 30 s at 94°C). As positive controls, aliquots of plasmid DNA containing previously (Leygue et al, 1996a) subcloned WT-PR (WT cont) or exon 6-deleted PR (D6 cont) sequences were amplified in parallel. PCR products were then separated on 6% polyacrylamide gels containing 7 M urea (PAGE). Following electrophoresis, the gels were dried and autoradiographed. The PCR product corresponding in size to D6-PR was subcloned and sequenced as previously described (Leygue et al, 1996b).

Quantification and statistical analysis

The approach used to evaluate the exon-deleted variant mRNA expression relative to WT mRNA has been previously validated for exon-deleted ER variant mRNAs (Daffada et al, 1994; Leygue

et al, 1996b). PCR co-amplification of WT and exon-deleted variant generates two bands whose ratio is constant with varying cycle number and is independent of initial input cDNA. This assay provides a semi-quantitative RT-PCR in which the internal control is the WT mRNA co-amplified and in which relative expression of variant mRNA can be determined for individual samples. Bands corresponding to D6-PR and WT mRNAs were excised from the gel and corresponding signals were subsequently measured after addition of 5 ml scintillant (ICN Pharmaceuticals, Inc., Irvine, CA, USA) by counting. The D6-PR signal was expressed as a percentage of the WT-PR signal. For each sample, three independent assays were performed and the mean determined. The statistical significance of differences in the relative levels of expression of D6-PR mRNAs was determined using the Mann-Whitney rank-sum test (two-sided).

RESULTS

Detection of D6-PR in all normal and tumour breast tissues

Total RNA from ten normal breast tissues and 17 breast tumour specimens was analysed by RT-PCR as described in Material and Methods using primers depicted in Figure 1. These primers were designed to allow the co-amplification of D6-PR and WT-PR mRNAs. Among the 17 tumours studied, nine had a high PR level (> 100 fmol mg⁻¹ protein) and eight had a low PR level (< 15 fmol mg⁻¹ protein), as

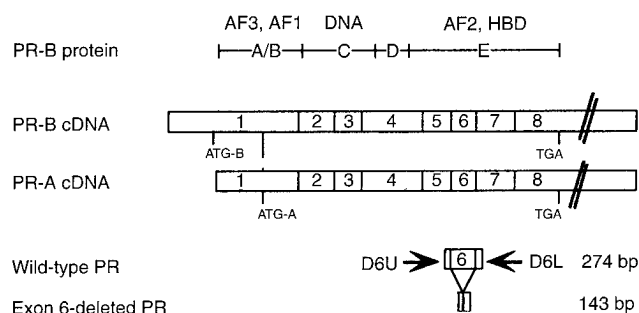


Figure 1 Schematic representation of PR-B protein, PR-B and PR-A cDNAs and primers used to co-amplify WT-PR and D6-PR variant cDNAs. PR cDNAs contain eight different exons coding for a protein divided into structural and functional domains (A-E). A/B region of the receptor contains two transactivating domains (AF3 and AF1). The C region contains the DNA binding domain whereas region E, which is involved in hormone binding, contains another transactivating domain (AF2). ATG-B and ATG-A are the translational start sites of PR-B and PR-A proteins respectively. TGA, stop codon. D6U and D6L primers allow co-amplification of 274 bp and 143 bp fragments corresponding to WT-PR and D6-PR mRNAs, respectively

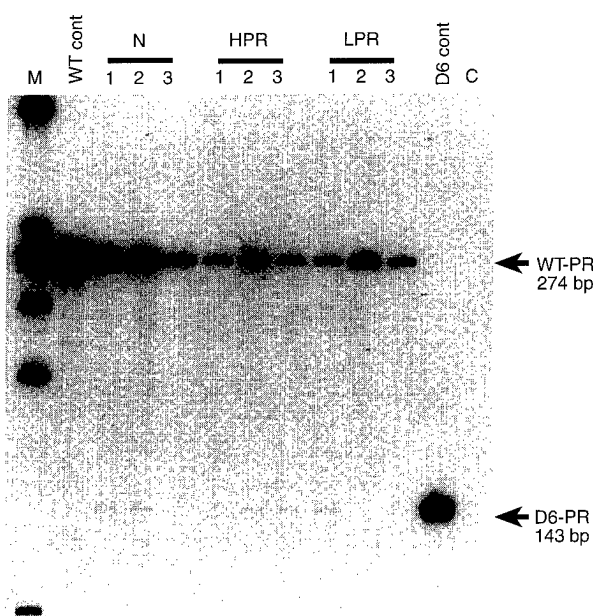


Figure 2 Detection of exon 6-deleted PR variant mRNA in all human breast samples. Total RNA extracted from normal (N1-3), high PR tumour (HPR1-3) and low PR tumour (LPR1-3) breast tissue samples was reverse transcribed and PCR-amplified as described in Material and Methods, using D6U and D6L primers. Radioactive PCR products were separated on a 6% acrylamide gel and visualized by autoradiography. Bands that migrated at 274 bp and 143 bp were identified as corresponding to WT-PR and exon 6-deleted PR variant mRNAs, respectively. Plasmids containing either WT-PR (WT cont) or exon 6-deleted PR (D6 cont) sequences were used as positive control. M: molecular weight marker (ϕ x 174 HaeIII digest, Gibco BRL, Grand Island, NY, USA). C: no cDNA added during the PCR reaction

determined by ligand binding assay. In each sample, two major bands that corresponded in size to that expected for WT-PR and D6-PR PCR products were obtained. Figure 2 presents a typical autoradiograph after one night's exposure. It should be noted that a longer exposure or addition of intensifying screens allowed the detection of D6-PR in both lanes N3 and LPR3 (data not shown). One should also note the presence, in samples where WT-PR signal is high (such as in lane N2, Figure 2), of some minor PCR products, that are not reproducibly obtained and therefore were not further characterized. The PCR product corresponding in size to D6-PR and reproducibly obtained was subcloned and subsequently sequenced. Sequence analysis showed the expected perfect junction between exon 5 and exon 7 (data not shown).

Comparison of D6-PR variant expression in normal and tumour tissues

The D6-PR variant mRNA expression relative to WT-PR was then evaluated in each sample. It has been previously demonstrated that the co-amplification of WT and exon-deleted variant transcripts led to the synthesis of two PCR products. Further, the ratio of the signals obtained from these two products could be used to compare relative exon-deleted variant expression within samples (Daffada et al, 1994; Leygue et al, 1996b). The signal corresponding to D6-PR was expressed as a percentage of the WT-PR signal and the mean of three different assays calculated (Figure 3). The level of exon 6-deleted variant mRNA relative to the WT-PR mRNA was found to be significantly ($P < 0.05$) lower in normal (median = 4.8%) than in neoplastic breast tissues having either high PR or low PR (median = 9.19% and median = 25.13% respectively). The significance became higher ($P < 0.01$) when the tumour subgroups were considered together (median = 13.86%). Moreover, even though the difference did not achieve statistical significance ($0.1 < P < 0.05$), D6-PR relative expression appeared lower in tumours with high PR levels (median = 9.19%) than in tumours with low PR levels (median = 25.13%).

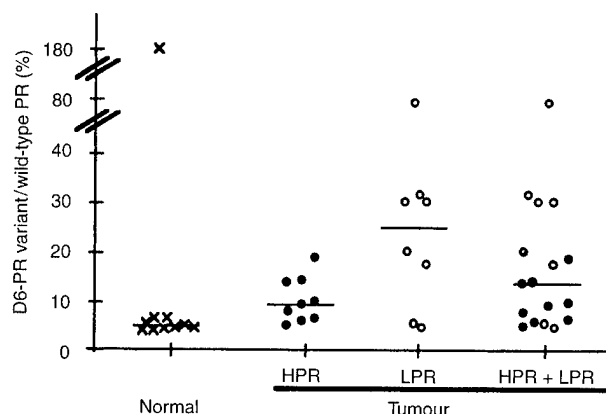


Figure 3 Comparison of D6-PR relative expression between normal and tumour samples. Total RNA extracted from ten normal breast samples (x), nine tumours with high PR (●, HPR) and eight tumours with low PR (○, LPR) was reverse transcribed and PCR-amplified as described in Material and Methods, using D6U and D6L primers. D6-PR corresponding signal was measured as described in Material and Methods and expressed as a percentage of wild-type PR corresponding signal. For each sample, the mean of three different experiments is indicated. Bars: medians

DISCUSSION

This study shows that the relative expression of D6-PR variant mRNA is altered between normal breast tissue and breast cancer tissue samples. The expression of D6-PR variant mRNA relative to WT-PR mRNA was investigated using a previously described semi-quantitative RT-PCR assay (Daffada et al, 1994; Leygue et al, 1996b). This assay allows the determination of the expression of PR variant mRNA relative to WT-PR mRNA using the very small amount of starting material provided by histopathologically well-characterized regions within human breast tissue.

It is important to establish whether or not this differential expression of D6-PR variant is maintained at the protein level. Anti-PR antibodies available to date (such as hPRa-7; Clarke et al, 1987), while able to recognize the predicted PR variant proteins, cannot provide any information on the unique primary sequence of the recognized molecule (Yeates et al, 1998). We are in the process of developing an antibody, raised against the predicted 12 specific C-terminal amino acids of the D6-PR protein (Leygue et al, 1996a) that could be used for the specific immunochemical detection of D6-PR variant protein within paraffin-embedded breast tissue sections or by Western blotting.

The recombinant D6-PR protein, has recently been shown to bind constitutively the progesterone receptor element (PRE) DNA consensus sequence and to exhibit dominant-negative activity on PR-A- and PR-B-induced transcription (Richer et al, 1998). Interestingly, a naturally occurring ER variant mRNA deleted in exon 7 and encoding an analogous truncated molecule lacking the hormone binding domain of the WT-ER can also act as a dominant-negative regulator of WT-ER, at least under some circumstances (Wang and Miksicek, 1991; Fuqua et al, 1992).

Relative levels of some of the ER variants were found to be increased during tumour progression. Exon 7-deleted variant mRNA level was shown to be higher in ER + PR – versus ER + PR + tumours (Fuqua et al, 1992). Exon 5-deleted ER variant mRNA expression was found higher in ER – PR + versus ER + PR + tumours (Fuqua et al, 1991) and was decreased in normal versus tumour breast tissues (Leygue et al, 1996b). It has thus been speculated that expression of these ER variants may be altered during breast tumorigenesis and progression and may have a role in progression from hormone dependence to independence in breast cancer (Murphy et al, 1997). This aspect of tumour progression consists of altered oestrogen signalling, the acquisition of resistance to the cytostatic effects of the anti-oestrogen tamoxifen and subsequently in the failure to respond to agents such as progestins and probably antiprogestins (RU 486) (Horwitz et al, 1995). The apparent lower relative expression of D6-PR in normal breast samples compared to tumour tissues, as well as in high PR tumours compared to low PR tumours, is therefore of interest, since normal tissue, high PR tumours and low PR tumours may represent steps in tumour progression which correlate with increasing relative D6-PR expression. In order to clarify such issues, larger numbers of samples require screening for D6-PR expression.

The measurement of PR is an important tool in clinical decision-making with respect to prognosis and treatment of human breast cancer. Furthermore, the level of PR expression provides important clinical information as shown by Clark et al (1983). As the use of enzyme-linked immunosorbent assays (ELISA) and immuno-histochemical assays for PR detection increases, it is likely that variant PR expression will interfere with these assays. Such

capability of variant forms of receptor to possibly interfere with immunohistochemical detection of the WT molecule has recently been demonstrated for ER (Huang et al, 1997 and unpublished data).

In conclusion, we show in this study that exon 6-deleted PR variant mRNA relative expression is increased during breast tumorigenesis. We speculate that PR variants may have a role in tumorigenesis and/or be a marker of breast cancer progression, as already suggested for ER variants.

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APPENDIX 9

**Leygue E, Huang A, Dotzlaw H, Stickles S, Watson PH,
Khan S, and Murphy LC**

Expression of estrogen receptor alpha variant mRNAs in normal human breast
tissue of women with and without breast cancer.

Submitted, Clin Cancer Res.

EXPRESSION OF ESTROGEN RECEPTOR ALPHA VARIANT mRNAs IN NORMAL HUMAN BREAST TISSUE OF WOMEN WITH AND WITHOUT BREAST CANCER¹

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Running title: ER variant mRNA expression in normal human breast tissue.

Key words: breast tumorigenesis, PCR, TP-PCR.

Abstract

The expression of three previously described estrogen receptor alpha (ER- α) variant mRNAs, that are deleted in exon 5 (ERD5), exon 7 (ERD7) or exon 3 to 8 (ERC4) sequences of the wild-type ER- α (WT-ER) mRNA, has been measured by reverse transcription polymerase chain reaction (RT-PCR) in the normal breast tissue from women with (cases, n=19) or without (controls, n=18) breast cancer. A significantly higher (Mann-Whitney rank sum test, $p<0.02$) expression of ERD5 variant mRNA relative to WT-ER mRNA was observed in the normal breast tissue of controls (median 20.35%, n=16) as compared to cases (median = 10.97%, n= 19). The levels of expression of ERD7 and ERC4 variant mRNAs were found to be similar in the normal breast tissue of both subgroups of patients. Our results suggest that ER signaling pathway in the normal breast tissue of women with breast cancer differs from that of women without. Whether these differences result from or pre-exist to the presence of breast cancer remains to be determined.

Introduction

Estrogens, that regulate the growth and the development of normal human mammary tissue, are also involved in breast tumor progression (1). Estrogen action is mainly mediated through two estrogen receptors, estrogen receptor alpha (ER- α)³ and estrogen receptor beta (2, 3). These two receptors, that belong to the steroid/thyroid/retinoic acid receptors super-family, share the same structural and functional domain composition (4-6), defined as regions A to F (see Fig. 1A). The receptors, through a ligand binding domain located within the E region, bind ligand which leads to conformational changes. These changes result in hormone/receptor complexes, which recognize estrogen responsive elements (ERE) located upstream of target genes through a DNA binding domain located within the C region of the receptor. Interactions between the transactivation domains located in the A-B (AF-1) and E (AF-2) regions of ERs, and accessory proteins ultimately lead to the modification of the transcription of these genes (7). Recently, Khan et al. demonstrated that the expression of ER- α , assessed by immunocytochemistry performed using an antibody recognizing the C-terminal extremity of ER- α , was different in the normal breast tissue of women with (cases) and without (controls) breast cancer (8). This study showed that the expression of ER- α is higher in the normal breast tissue of control women during the follicular phase of the menstrual cycle, whereas an inverse trend is observed during the luteal phase. Indeed, the normal

breast tissue of case women expresses a higher level of ER- α than that of control women during the luteal phase (8).

Discrepancies have been observed in the immunocytochemical assessment of ER- α protein expression within breast tissues, depending on whether the antibody used recognizes a C-terminal or a N-terminal epitope of the wild-type ER molecule (9). It has been demonstrated that these discrepancies correlated with the presence in the sample studied, of several variant mRNAs, that would encode truncated ER-like proteins missing some of the C-terminal functional domains of the wild-type receptors (10). Among these potential variant forms of ER- α , several could interfere with ER- α signaling pathways. Indeed, exon 5-deleted (ERD5) and exon 7-deleted ER- α (ERD7) variant proteins have been shown, *in vitro*, to exhibit a constitutive transcriptional (11) and a dominant negative activity (12) on ER- α , respectively. We hypothesized that the differences observed by Khan et al. in the expression of ER- α between the normal breast tissue of cases and controls could in part result from the presence of particular truncated ER-variant molecules. To address this question, quantitative reverse transcription polymerase chain reaction assays have been used to assess the expression of the most commonly expressed ER- α variant mRNAs (13) able to encode ER-like truncated protein (i.e ERD5, ERD7 and ERC4 mRNAs) in the normal breast tissue of controls and cases during the luteal phase of the menstrual cycle.

Materials and methods

Normal human breast tissues.

Nineteen case and eighteen control subjects in the luteal phase of their menstrual cycle at the time of surgery were selected from the previously described cohort analyzed by Khan et al. (8). Briefly, subjects were recruited from the Breast Care Center at University Hospital, Syracuse, NY. Case subjects were women with newly or previously diagnosed *in situ* (n = 6) or invasive (n = 13) breast cancer who required further surgery. Six case samples were obtained from mastectomies whereas thirteen samples came from biopsies. Control subjects were women without a prior history of breast cancer who required diagnostic breast biopsy, but proved not to have breast cancer. The lesions observed in control women were fibroadenoma (n = 8), fibrocystic diseases (n = 3), hyperplasia (n = 2), fibrosis (n = 3) and intraductal papilloma (n = 1). The remaining control sample was obtained from reduction mammoplasty. The phase of the cycle was established by questioning patients at the time of surgery. The presence of normal ducts and lobules as well as the absence of any atypical lesion in all normal tissue

specimens was confirmed by histopathological examination of H&E stained tissue sections. Total RNA was extracted from frozen breast tissue sections using Trizol™ reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions, and quantified spectrophotometrically. One µg of total RNA was reverse-transcribed in a final volume of 25 µl as previously described (14). The quality of the cDNAs obtained was checked by PCR amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as described previously (17).

Primers and PCR conditions.

The primer set which co-detected WT-ER and ERD5 cDNAs consisted of ER4U primer (5'-CAGGGGTGAAGTGGGGTCTGCTG-3'; sense; located in WT-ER exon 4; 1060-1082) and ER6L primer (5'-ATGCGGAACCGAGATGATGTAGC-3'; antisense; located in WT-ER exon 6; 1520-1542). This primer set allowed amplification of 483 bp and 344 bp fragments corresponding to WT-ER and ERD5 cDNAs, respectively. The primer set designed to co-detect WT-ER and ERD7 cDNAs consisted of ER5U primer (5'-TCCTGATGATTGGTCTCGTCTGG-3'; sense; located in WT-ER exon 5; 1389-1411) and ER8L primer (5'-CAGGGATTATCTGAACCGTGTGG-3'; antisense; located in WT ER exon 8; 2035-2057). This primer set allowed amplification of 668 bp and 484 bp fragments corresponding to WT-ER and ERD7 cDNAs, respectively. ER2U primer (5'-TGTGCAATGACTATGCTTCA-3', sense, located in WT-ER exon 2; 792-811), ER3L primer (5'-GCTCTTCCTCCTGTTTTTAT-3', antisense, located in WT-ER exon 3; 921-940) and ERC4L primer (5'-TTTCAGTCTTCAGATACCCAG-3', antisense; 1315-1336, as numbered in Dotzlaw *et al.*, 15) allowed amplification of a 536 bp and 148 bp fragments corresponding to ERC4 and WT-ER cDNA, respectively. WT-ER positions given correspond to WT-ER published cDNA sequence (2). PCR conditions used to detect ERD5, ERD7 and ERC4 cDNAs were as previously described (14, 16-17). Briefly, PCR amplifications were performed in a final volume of 10 µl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer, 0.2 units of Taq DNA polymerase (GIBCO-BRL) and 1 µCi of dCTP [α -³²P] (3000 Ci/mmol, ICN Pharmaceuticals Inc, Irvine, California). Each PCR consisted of 30 cycles (1 min at 94°C, 30 sec at 60°C and 1 min at 72°C) using a Thermocycler (Perkin Elmer). Four µl of the reaction were then denatured by addition of 6 µl of 80% formamide buffer and boiling

before electrophoresis on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and exposed overnight to Kodak XAR Film at -70°C.

Quantification of RT-PCR products and statistical analysis.

Bands corresponding to the variant ER cDNA and WT-ER cDNA were excised from the gel and counted in a scintillation counter as described previously (16, 17). ERD5, ERD7 and ERC4 signals were then expressed as a percentage of the WT-ER signal. Indeed, we and others have previously showed that for each sample, the ratios of ERC4/WT-ER, ERD5/WT-ER signal and ERD7/WT-ER signal remained constant and independent of the number of PCR cycles or initial cDNA input (16, 17). Three and in most cases four independent PCR reactions were performed and the mean determined. Only samples having measurable levels of WT-ER mRNA in at least two experiments were included in the statistical analysis. The statistical significance of differences in the relative levels of expression of any single ER- α mRNA variant between normal breast tissue from cases and controls was tested using the Mann-Whitney rank sum test, two sided.

Results

Relative expression of ERD5 mRNA in normal breast tissue from case and control subjects.

Total RNA was extracted from frozen tissue sections corresponding to the normal breast tissue obtained from nineteen cases and nineteen controls during the luteal phase of their menstrual cycle. Reverse transcription and polymerase chain reaction were performed as described in the Materials and Methods section. Primers used in the PCR consisted of primers annealing to exon 4 and exon 6 sequences, as illustrated in Fig.1A. PCR amplification performed using these primers, has previously been reported to produce 483 bp and 344 bp fragments corresponding to WT-ER and ERD5 mRNA, respectively (17). As shown in Fig.2A, WT-ER and ERD5 PCR products were observed in the normal breast tissue from both case and control women. It has previously been demonstrated that the ratio between the two PCR products was directly related to the ratio of the corresponding initial mRNAs (17). The expression of ERD5 mRNA relative to WT-ER mRNA was measured as described in the Material and Methods section in the 16 controls and 19 cases where WT-ER PCR products were reproducibly detected. Fig.2B shows the results obtained. A significantly (Mann-Whitney rank sum test, $p = 0.019$) higher

relative expression of ERD5 mRNA was observed in the normal breast tissue of controls (n = 16, median = 20.35%) as compared to that of cases (n = 19, median = 10.97%).

Relative expression of ERD7 mRNA in normal breast tissue from case and control subjects.

Normal breast tissue cDNAs corresponding to case and control women were PCR amplified using primers depicted in Fig.1A and annealing with exon 5 and exon 8 sequences. As shown Fig. 3A, two PCR products, migrating at an apparent size of 668 bp and 484 bp were obtained. These PCR products have previously been shown to correspond to WT-ER cDNA and ERD7 cDNA, respectively (17). The expression of ERD7 relative to WT-ER was measurable in 15 control and 18 case women. Results presented Fig.3B show that no statistically significant difference (Mann-Whitney rank sum test, $p = 0.624$) exists in the relative expression of ERD7 measured in controls (n = 15, median = 42.63%) and in cases (n=18, median = 49.97%)

Relative expression of ERC4 mRNA in normal breast tissue from case and control subjects.

In order to investigate the relative expression of ERC4 mRNA within normal breast tissue from case and control women, a recently described triple-primer PCR has been used (14, 16). This assay relies on the co-amplification of WT-ER and ERC4 cDNAs using three primers in the PCR reaction. As shown Fig.1A-B, one primer (ER2U) is able to anneal to common exon 2 sequences whereas the two other primers anneal specifically to the WT-ER exon 3 (ER3L) and the ERC4 unique sequence (ERC4L), respectively. It has been previously demonstrated that the ratio between the two PCR products obtained, 536bp and 149 bp which correspond to ERC4 and WT-ER cDNAs, respectively, is directly related to the ratio of the respective initial RNA (14, 16). As expected, ERC4 and WT-ER PCR products were detectable in the normal breast tissue of both cases and controls (Fig. 4A). No statistically significant difference (Mann-Whitney rank sum test, $p = 0.980$) was observed between the relative expression of ERC4 mRNA in the normal breast tissue of control (n = 16, median = 6.58%) and case (n = 19, median = 7.23%) women.

Discussion

Recently, Khan et al. reported differences in the expression of ER- α between the normal breast tissue of women with (cases) and without (controls) breast cancer (8). Indeed, in the luteal phase of their menstrual cycle, controls

expressed lower ER- α levels in their normal breast tissue than cases. In this study, Khan et al. evaluated ER- α expression by immunocytochemistry performed using a C-terminal-targeted antibody.

It has previously been demonstrated that the assessment of ER- α levels in human breast tissues by immunocytochemistry can give discrepant results, depending on whether the antibodies used to detect ER recognized the C-terminal or the N-terminal extremity of the protein (9). Indeed, when ER- α signals obtained with the two kinds of antibodies were compared, tumors fell into two groups, with one group having similar ER- α levels (consistent tumors), as measured with the two antibodies and the other (inconsistent tumors) having higher levels when the amino-terminal-targeted antibody was used (9). It was shown that these discrepancies in ER- α levels correlated with the presence in the inconsistent breast tumor group of higher levels of ER- α variant mRNAs able to encode truncated ER-like molecules (10).

It was therefore important to determine whether the low level of ER- α expression observed by Khan et al. in the normal breast tissue of control patients during their luteal phase, correlated with an increase in ER- α variant mRNAs encoding truncated ER-like proteins.

A PCR-based assay, that allows the evaluation of the relative pattern of expression of all exon deleted ER- α variant transcripts present in any individual breast tumor sample, has recently been described (18, 19). This so called long-range PCR assay is based on the competitive amplification of wild-type and exon deleted ER- α variant cDNAs using primers annealing within exons 1 and 8. Long-range PCR failed to give reproducible results when performed on normal breast tissue samples (our unpublished observations). The lack of reproducible signals, already observed for ER negative breast tumor samples, as assessed by ligand binding assay (19), is likely to result from the low level of expression of ER- α in normal breast tissues. Indeed, the low number of WT-ER cDNA molecules does not allow the corresponding long fragment PCR product (1381 bp) to be reproducibly and efficiently amplified during the first cycles of the PCR (19).

Because it was not possible to analyze the exon-deleted ER- α variant mRNA population in its entirety by long-range PCR, the expression of the three most commonly studied ER- α variant mRNAs encoding C-terminally truncated ER-like proteins (i.e ERD5, ERD7 and ERC4 variant mRNAs) was investigated by targeted PCR assays. It should be stressed that these assays are performed using primers spanning only a limited region of the

recognized cDNAs. Therefore, while the relative proportion of the regions spanned by the set of primers can be determined, no data are provided on the other regions of the recognized cDNAs. Indeed, it is now clear that more than one modification can occur in variant transcripts (13). Thus signals attributed to the exon 7-deleted ER- α variant mRNA detected by targeted PCR performed using primers in exon 5 and 8 may also include contributions from a variant deleted in both exon 4 and 7, previously identified by Madsen et al. (20). Similarly, using the same primers, the signal attributed to WT-ER mRNA results in fact from the sum of the signals corresponding to WT-ER, exon 3 deleted, exon 4 deleted and all other ER- α like mRNAs sharing intact exon 5 to 8 regions. This important limitation concerning the real identity of the cDNA molecules detected using targeted PCR has to be kept in mind when trying to extrapolate the data at the protein level.

The relative expression of ERD5, ERD7 and ERC4 ER variant mRNAs has been evaluated in the normal breast tissue of case and control women, obtained during the luteal phase of their menstrual cycle. As opposed to the results obtained using long-range PCR, a signal corresponding to WT-ER cDNA was reproducibly obtained for most of the samples, even though the concentration of the target cDNA is known to be low. This is consistent with a much higher efficiency of the PCR when shorter PCR fragments (smaller than 700 bp) are amplified. The expression of ERD5 mRNA was significantly lower in the normal breast tissue of women with breast cancer as compared to that of women without. In contrast, no significant difference in the relative expression of ERD7 and ERC4 mRNAs between the two subgroups was observed. As underlined earlier in the text, even though the apparent decrease in ERD5 mRNA expression within cases can effectively result from a change in ERD5 mRNA expression, it might also result from a change in other ER- α variant mRNA molecules recognized by the two primers. Indeed, an increase in such ER- α variant mRNA molecules in the cases, resulting in an increase of the signal attributed to WT-ER mRNA signal, could explain the decrease in the apparent ERD5 signal. Similarly, the apparent similar expression of ERD7 (or ERC4) mRNAs in the two subgroups only reflects an absence of change in the balance between the sum of the different ER- α like cDNA molecules, visualized through the two bands detected.

In the light of the results obtained, one might hypothesize that the lower expression of ER- α protein in the normal breast tissue of control women results from an increase in ERD5 expression in these samples. ERD5 protein has a theoretical molecular mass of 41kDa. Because several other ER variant mRNAs able to encode ER-like proteins

with similar molecular masses have been described (13), only antibodies recognizing ERD5 C-terminal specific amino-acids will allow the question of the differential relative expression of ERD5 by Western blot analysis to be addressed. Alternatively, since WT-ER- α expression may be upregulated in cases, the decreased relative expression of ERD5 suggests an altered regulation of expression of the two transcripts. No data are available to date in which the regulation of ER- α variant mRNA expression has been investigated.

Changes in the relative expression of ERD5 mRNA, monitored using a similar assay, have previously been shown to occur during breast tumorigenesis and tumor progression. Indeed, the relative expression of ERD5 mRNA was found lower in normal reduction mammoplasty samples as compared to ER positive invasive ductal carcinoma (17). As well, an increased expression of ERD5 mRNA has been observed in the tumor component of breast tumor samples, as compared to their matched adjacent normal breast tissue (our unpublished data). Moreover, a higher expression of ERD5 mRNA in ER negative/Progesterone receptor positive (as determined by ligand binding assay) breast tumors than in ER positive/Progesterone receptor positive tumors has been described (11). Functional analysis performed in *in vitro* systems revealed that ERD5 protein displays ligand-independent transcriptional activity (11, 21). This observation led to the speculation that an increase in ERD5 mRNA, possibly paralleled with an increase in ERD5 protein, could be involved in the hormone independent phenotype occurring during tumor progression. However, evidence suggests that ERD5 and other variants are expressed with WT-ER. It has been shown that when ERD5 is expressed with WT-ER, it has an inhibitory effect on WT-ER protein activity (11, 21). Therefore, ERD5 may have a modulator activity that depends on the relative expression of the two proteins as well as the need for them to be expressed within the same cells. However, as mentioned earlier, one should be very careful in extrapolating targeted RT-PCR data to protein analysis.

Whether or not the change observed in ERD5 mRNA expression reflects a modification of the expression of ERD5 mRNA is unknown, however the data support a modification of the balance existing between the different ER- α like mRNAs present in the normal breast tissue of cases and controls. This suggests that ER- α signaling pathway may differ in the normal breast tissue of the two groups. Interestingly, the normal breast tissue adjacent to breast tumor has recently been shown to be able to present some genetic abnormalities (22). Indeed, Deng et al. reported a loss of heterozygosity in 3p22-25 in morphologically normal lobules adjacent to breast cancers. Because some of the case samples in this study were obtained from biopsies and therefore came from normal

tissue adjacent to the tumor, one cannot exclude that the difference in the expression of ERD5 mRNA between cases and controls results from the vicinity of tumor tissue. Whether or not the differences observed pre-exist to or result from the presence of breast cancer remains to be determined.

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Footnotes:

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³ The abbreviations used are: ER, estrogen receptor; ERD5, exon 5 deleted estrogen receptor variant; ERD7, exon 7 deleted estrogen receptor variant, ERC4, estrogen receptor variant deleted in exon 3 to 8 sequences; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; TP-PCR, triple primer polymerase chain reaction.

Figures legend

Fig. 1

A. Schematic representation of WT-ER cDNA and primers used to detect ERD5 and ERD7 variant mRNAs. ER cDNA contains 8 different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in ligand binding (LBD) and another trans-activating function (AF-2). ER4U and ER6L primers allow amplification of 483 bp and 344 bp fragments corresponding to WT-ER and ERD5 cDNAs, respectively. ER5U and ER8L primers allow amplification of 668 bp and 484 bp fragments corresponding to WT-ER and ERD7 cDNAs, respectively. ER2U and ER3L allow amplification of a 149 bp fragment corresponding to WT-ER cDNA.

B. Schematic representation of ERC4 variant cDNA and primers used to detect this variant. ERC4 cDNA contains the first 2 exons of WT-ER cDNA followed by sequences unrelated to WT-ER sequence (17). ER2U and ERC4L primers allow amplification of a 536 bp fragment corresponding to ERC4 cDNA.

Fig. 2

ERD5 mRNA relative expression in the normal breast tissue of women with (case) and without (control) breast cancer. **A.** Total RNA was extracted from frozen tissue sections obtained from the normal breast tissue of nineteen controls (C) and nineteen cases (K) in the luteal phase of the menstrual cycle. RT-PCR was performed as described in the Materials and Methods section using ER4U and ER6L primers in the PCR. Radiolabelled PCR products, separated on polyacrylamide gels, have been shown to correspond to WT-ER and ER-D5 mRNA. **B.** The expression of ERD5 mRNA relative to WT-ER mRNA (ERD5 % WT-ER) was measured in the normal breast tissue of 16 control and 19 case women as described in the Materials and Methods section. The median of each subgroup is indicated by a horizontal bar. p value: Mann-Whitney rank sum test, two sided.

Fig. 3

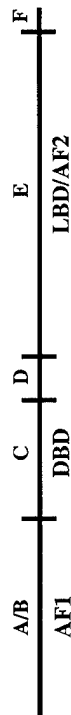
ERD7 mRNA relative expression in the normal breast tissue of women with (case) and without (control) breast cancer. **A.** Total RNA was extracted from frozen tissue sections obtained from the normal breast tissue of nineteen control (C) and nineteen case (K) pre-menopausal women in the luteal phase of their menstrual cycle. RT-PCR was performed as described in the Materials and Methods section using ER5U and ER8L primers in the PCR. The two radiolabelled PCR products, separated on polyacrylamide gels, have been shown to correspond to WT-ER and ER-D7 mRNA. **B.** The expression of ERD7 mRNA relative to WT-ER mRNA (ERD7 % WT-ER) was measured in the normal breast tissue of 15 control and 18 case women as described in the materials and Methods section. The median of each subgroup is indicated by a horizontal bar. p value: Mann-Whitney rank sum test, two sided.

Fig. 4

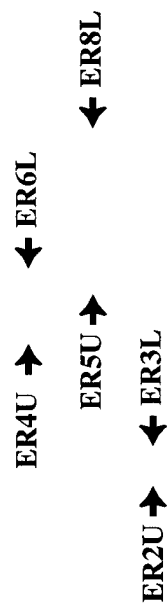
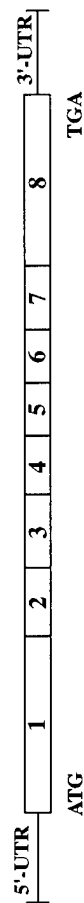
ERC4 mRNA relative expression in the normal breast tissue of women with (case) and without (control) breast cancer. **A.** Total RNA was extracted from frozen tissue sections obtained from the normal breast tissue of nineteen control (C) and nineteen case (K) pre-menopausal women in the luteal phase of their menstrual cycle. RT-PCR was performed as described in the Materials and Methods section using ER2U, ER3L and ERC4L primers in the PCR. The two radiolabelled PCR products, separated on polyacrylamide gels, have been shown to correspond to ERC4 and WT-ER mRNA. **B.** The expression of ERC4 mRNA relative to WT-ER mRNA (ERC4 % WT-ER) was measured in the normal breast tissue of 16 control and 19 case women as described in the Materials and Methods section. The median of each subgroup is indicated by a horizontal bar. p value: Mann-Whitney rank sum test, two sided.

A

WT-ER protein



WT-ER cDNA



B

ERC4 cDNA

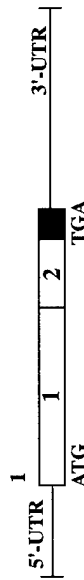


Figure 1

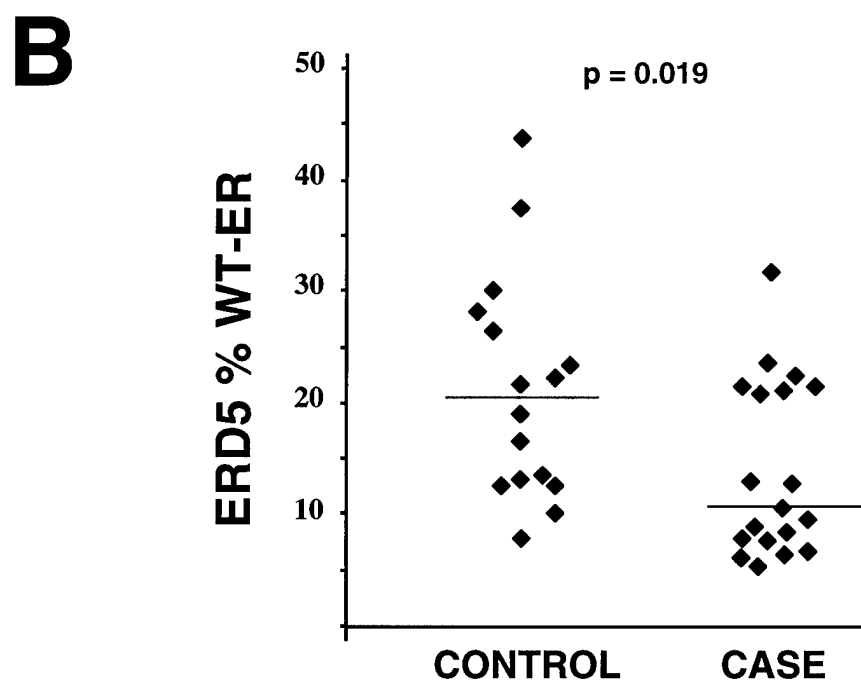
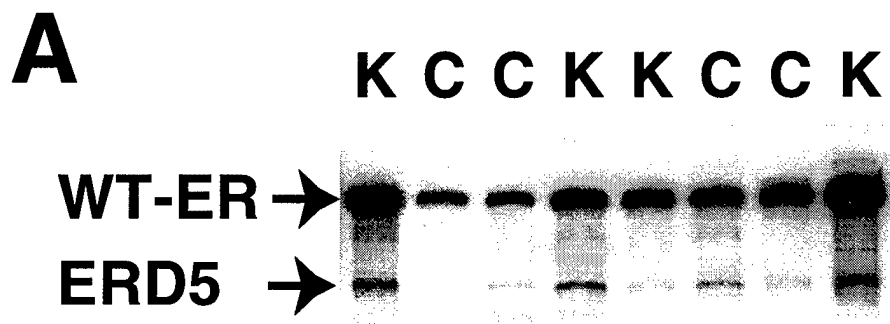


Figure 2

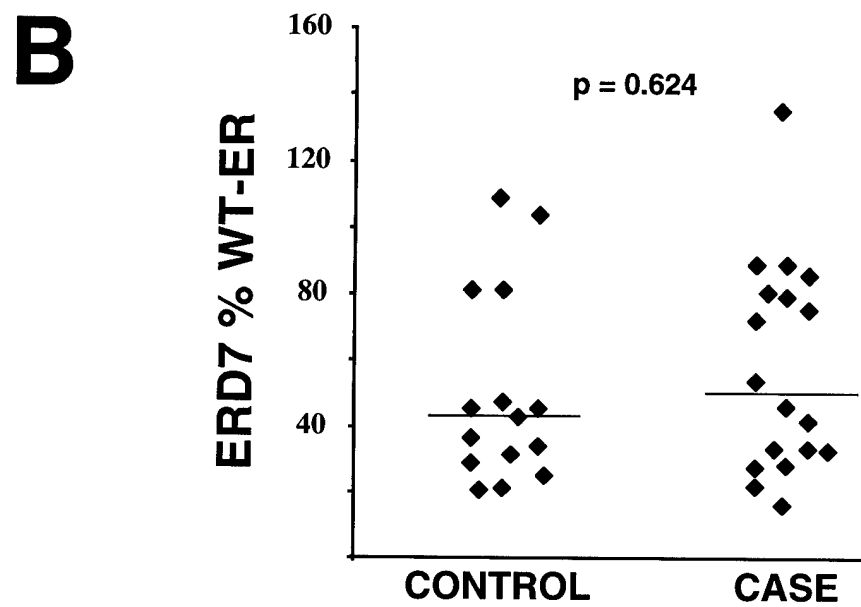
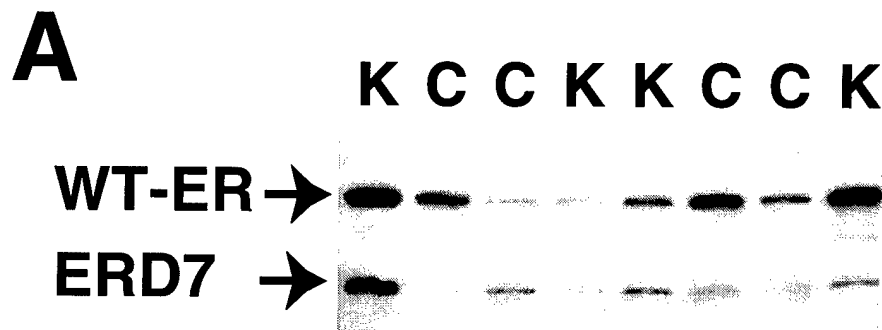


Figure 3

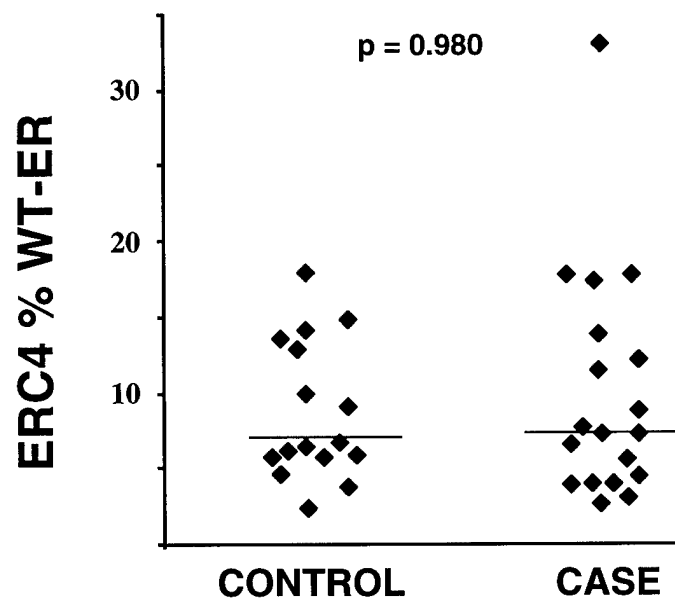
A**B**

Figure 4

APPENDIX 10

Dotzlaw H, **Leygue E**, Watson PH, and Murphy LC

Expression of estrogen receptor-beta in human breast tumors.

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EXPRESSION OF ESTROGEN RECEPTOR-*BETA* IN HUMAN BREAST TUMORS

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ABSTRACT

The expression of a recently described novel estrogen receptor, ER- β , was detected in several human breast tumor biopsy samples and several human breast epithelial cell lines using reverse transcription and polymerase chain reaction (RT-PCR) analysis. Cloning and sequencing of the PCR product from a breast tumor confirmed the identity of the sequence with that of the ER- β mRNA previously reported in human testis. The expression of ER- β was not correlated with that of ER- α , and both ER- α positive and ER- α negative cell lines expressed ER- β mRNA. However, some breast tumors and some cell lines coexpress ER- β and ER- α mRNA. Our data support a possible role for ER- β in human breast cancer.

Estrogen signal transduction plays an important role in both normal and neoplastic mammary tissue (1). The principal mechanism by which the effects of estrogen are mediated in either normal or neoplastic target cells is via an initial interaction with the estrogen receptor (ER), a member of the steroid/thyroid/retinoid receptor gene superfamily (2). Recently, a novel ER, referred to as ER- β was cloned and characterized from human testis (3) and its rat homolog was cloned and characterized from rat prostate (4). The ER- β protein has similarities to the classical ER referred to as ER- α , in terms of structure and function. Both of these proteins have a high degree of conservation of the DNA and ligand binding domains (3), while the A/B, hinge (D) and F domains are not conserved (3,4). Transient expression assays have shown that ER- β can bind estradiol and can transactivate estrogen regulated reporter genes, although less efficiently than ER- α , and antiestrogens can inhibit this effect (3,4). Further, the tissue distribution of ER- α and ER- β although not identical appears to overlap in some cases (3,4). Therefore ER- β may be involved independently in estrogen signal transduction in some tissues but in other tissues may contribute with ER- α in estrogen signal transduction.

Estrogen has an important role in human breast cancer, however, perturbations of ER signal transduction are thought to contribute to tumor progression and the eventual development of a hormone-independent and more aggressive phenotype (5-7). The expression

of ER- β in normal or neoplastic mammary tissue has not been reported, and it is important to determine if ER- β is expressed in breast cancers and therefore could potentially contribute to estrogen signal transduction in this tissue.

Materials and Methods

Human tissues, cell lines and RNA extraction.

Forty human breast tumor specimens left-over from steroid receptor assays were obtained from the Manitoba Breast Tumor Bank. Fourteen tumors were ER negative (ER < 3 fmol/mg protein), with progesterone receptor (PR) values ranging from 0 to 19.9 fmol/mg protein (median: 11.85 fmol/mg protein). Twenty six tumors presented ER levels ranging from 4.7 to 304 fmol/mg protein (median: 33.5 fmol/mg protein) and PR levels ranging from 4.1 to 764 fmol/mg protein (median: 50.5 fmol/mg protein). Total RNA was extracted using the guanidinium thiocyanate/cesium chloride method (8) as previously described (9). The human testis sample was obtained through the Manitoba Breast Tumor Bank and the MCF 10A1, MDA MB 231, T-47D and T-47D-5 cell lines were grown as previously described (10, 11). Total RNA from the cell lines and testis sample was extracted using Trizol reagent (Gibco/BRL) according to the manufacturers instructions, and the integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (9).

RT-PCR and Primers.

Total RNA (1.5 μ g per reaction), denatured at 65°C for 5 min, was reverse transcribed in a final volume of 15 μ l containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 μ M random hexamers (Gibco/BRL) and 150 units M-MLV reverse transcriptase (Gibco/BRL). The reaction was allowed to proceed for 60 min at 37°C and was then terminated by heating at 90°C for 5 min.

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One μ l of this reaction was amplified by PCR in a final volume of 25 μ l (if analyzed on 1.8% agarose gels stained with ethidium bromide) or 10 μ l (if incorporating [α - 32 P] dCTP and analyzed on 6% urea-PAGE), containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTPs, 4 ng/ μ l each primer and 0.02 unit/ μ l of Taq DNA Polymerase (Gibco/BRL).

The primers for ER- α were: ER- α upper (sense) 5' - CAG GGG TGA AGT GGG GTC TGC TG - 3' (priming site in exon 4, nucleotides 1060 - 1083 as numbered in reference 13); ER- α lower (antisense) 5' - ATG CGG AAC CGA GAT GAT GTA GC - 3' (priming site in exon 6, nucleotides 1520 - 1543). The PCR conditions were 25 cycles of 1 min 94°C, 30 sec 60°C, and 1 min 72°C, and 20 μ l of the PCR reactions were electrophoresed in agarose gels (1.8%) and visualized by ethidium bromide staining.

The primers used to amplify ER- β cDNA were: ER- β upper (sense) 5' - TGC TTT GGT TTG GGT GAT TGC - 3' (nucleotides 1164 - 1185 as numbered in reference 3); ER- β lower (antisense) 5' - TTT GCT TTT ACT GTC CTC TGC - 3' (nucleotides 1402 - 1423). The PCR conditions were 1 min 94°C, 30 sec 58°C, and 30 sec 72°C, for 30 cycles. PCR was done in the presence of [α - 32 P] dCTP (3000 Ci/mmol, 1 μ Ci per 10 μ l reaction), and 4 μ l of the reaction separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and autoradiographed. In some cases, the PCR was done in the absence of radioactivity for 40 cycles, and 20 μ l of the PCR reactions were electrophoresed in agarose gels (1.8%) and visualized by ethidium bromide staining.

In order to control for errors in input of cDNA used in PCR reactions, amplification of the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel using GAPDH primers (sense 5' - ACC CAC TCC TCC ACC TTT G - 3'; antisense 5' - CTC TTG TGC TCT TGC TGG G - 3') for 25 cycles of 1 min 94°C and 30 sec 52°C. PCR products were separated on agarose gels (1.8%) and visualized by ethidium bromide staining. All PCR reactions were performed at least twice in separate experiments.

PCR products from human testis and an appropriate breast tumor thought to represent ER- β were subcloned into the cloning vector, pGEM-T Easy (Promega) as previously described (12). Double stranded mini-prep DNA from two independent clones from each tissue was sequenced using a T7 Sequencing kit (Pharmacia) following the manufacturers protocol.

Results

Forty human breast cancer biopsies were analyzed for the expression of ER- β using radioactive RT-PCR as described above. It has previously been shown that the human testis expressed ER- β mRNA at relatively high levels, and consequently RNA extracted from a sample of non-malignant human testis was used as a positive control. A 259 bp PCR product of varying intensity was detected in 70% of the breast biopsy samples analyzed (Figure 1, panel A). Several tumors displaying high, intermediate and low levels of ER- β

expression using the radioactive PCR were reanalyzed using 40 cycles in a non-radioactive PCR. A 259 bp band equivalent to that found in the testis was detected in tumors displaying a strong signal in the radioactive PCR, while little if any product was detected in those tumors displaying intermediate and low signals in the radioactive PCR (Figure 1, panel B). The 259 bp signal is unlikely to result from amplification of contaminating genomic DNA, as the primers used were chosen to prime in what has been suggested to be separate exons (3). Moreover, an equivalent signal was obtained using cDNA in which the RNA had been treated with DNase I prior to reverse transcription (data not shown).

The 259 bp DNA fragments from the testis and a breast tumor sample in which a strong ER- β signal was detected were subcloned and sequenced. The tumor sequence was identical to the testis sequence, and matched that previously published for the human ER- β mRNA (3).

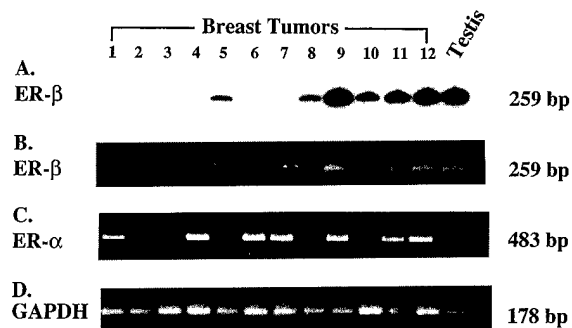


FIG. 1. A: Radiolabelled RT-PCR products using primers for ER- β and 30 cycles of PCR, separated on 6% PAGE containing 7M urea. Twelve human breast cancer biopsy samples and a positive control from a human testis sample are shown. The exposure was overnight with an intensifying screen. B: The same panel of human breast cancer biopsy samples and the human testis sample amplified with ER- β primers for 40 cycles of PCR separated on 1.8% agarose and stained with ethidium bromide. C: RT-PCR products using primers for ER- α from tumors and the testis sample shown in panels A and B separated on 1.8% agarose and stained with ethidium bromide. D: Expression of GAPDH in the tumor and testis samples shown in panels A, B and C.

Expression of ER- α mRNA in the testis and breast tumor samples was investigated using RT-PCR. The expected 483 bp DNA fragment was detected in 90% of the breast cancer biopsy samples by ethidium bromide staining (Figure 1, panel C) but no ER- α mRNA was detected in the testis sample (Figure 1, panel C). No correlation was seen between ER- α and ER- β

mRNA expression in the breast cancer biopsy samples. However, it was apparent that both genes could be expressed within the same tumor sample in some cases (see tumor samples in Figure 1, lanes 9,11,12).

To determine if the differences in level of detection were due to errors in input of cDNA used in PCR reactions, amplification of the ubiquitous GAPDH cDNA was performed in parallel using GAPDH primers (Figure 1, panel D). The results suggest similar levels of GAPDH in all samples analyzed.

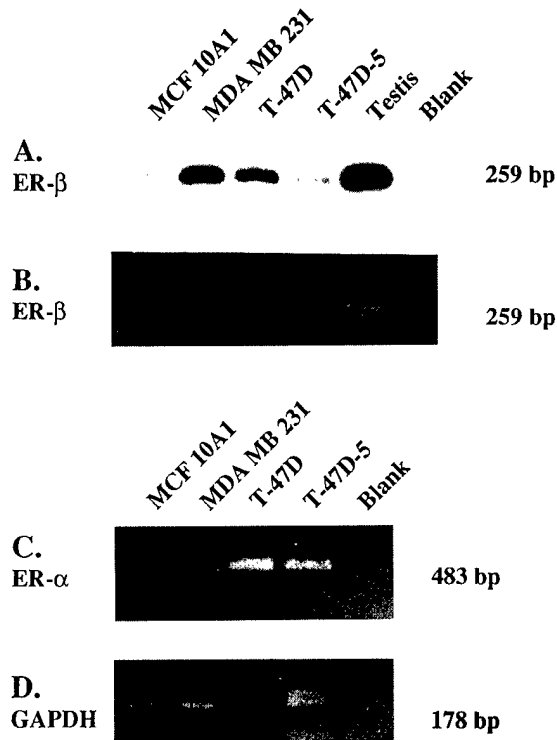


FIG. 2. A: Radiolabelled RT-PCR products using primers for ER- β and 30 cycles of PCR, separated on 6% PAGE containing 7M urea. Four human breast epithelial cell lines and a testis positive control are shown. The exposure was overnight with an intensifying screen. B: The four human breast cell lines shown in panel A amplified for 40 PCR cycles using primers for ER- β separated on 1.8% agarose and stained with ethidium bromide. C: Expression of ER- α in the human breast cell lines shown in panels A and B using RT-PCR. D: Amplification of GAPDH in the four epithelial cell lines shown in panels A, B and C.

The heterogenous nature of human breast tumor samples with respect to cell type (normal and neoplastic breast epithelial cells, normal stroma, myoepithelial cells, infiltrating lymphocytes) made it difficult to determine if the ER- β mRNA was ex-

pressed exclusively in tumor cells. To address this issue we analyzed the expression of ER- β mRNA in several human breast epithelial cell lines, including breast cancer cell lines. The expected 259 bp band was detected at varying levels in all breast epithelial cell lines following autoradiography of radiolabelled RT-PCR products (Figure 2, panel A), and was detected by ethidium bromide staining of nonradioactive RT-PCR products obtained from T-47D and MDA MB 231 cells (Figure 2, panel B).

ER- β mRNA was detected in cell lines which were both ER- α positive (T-47D, T-47D-5) and ER- α negative (MDA MB 231, MCF 10A1). ER- α mRNA expression was determined by RT-PCR (Figure 2, panel C). Differences in signal are unlikely to be due to differences in input cDNA as shown by the equivalent GAPDH signal observed in all samples (Figure 2, panel D).

Discussion

The data presented in this paper provide evidence for the expression of the ER- β gene in human breast epithelial cells. Our results are the first, to our knowledge, to address the issue of ER- β expression in either normal or neoplastic breast tissue or cells. ER- β mRNA was detected in both human breast tumor biopsy samples and human breast epithelial cell lines growing in culture. The level of expression of this gene appeared to vary amongst tumor samples and between cell lines, but the expression was not correlated with the expression of ER- α mRNA. Indeed both ER- α positive (T-47D) and ER- α negative (MDA MB 231) cell lines, as determined by ligand binding assays (14) and RT-PCR analysis (Figure 2, panel C), were found to express relatively high levels of ER- β mRNA. Interestingly, the non-tumorigenic, apparently 'normal' human mammary epithelial cell line, MCF 10A1 (11), contained detectable ER- β mRNA suggesting the possibility that ER- β may be expressed in normal human mammary epithelial cells. The ER- β has been shown to have some functional similarities to the ER- α in that it can bind estradiol-17 β and activate an ERE-regulated reporter gene construct, and antiestrogens can inhibit ER- β activity in these assays (3,4). However, reduced potency of estrogen activation of ER- β with respect to ER- α was noted (3), and since marked differences between these two ERs in the A/B, hinge (D) and F domains exist the assay systems previously used may not be optimal for ER- β in terms of the cell type, the promoter and possibly the ligand (3). The detection of relatively high levels of ER- β mRNA in MDA MB 231 breast cancer cells, which have previously been shown to be ER- α negative by ligand binding assays and in this paper by RT-PCR analyses, questions the

functional significance of ER- β expression in these cells at least with regard to mediating an 17 β -estradiol signal. However, the lack of any clearly defined function for this protein as well as possible different ligand preferences (15) severely limits the interpretation of such data.

Although we found no correlation between ER- α and ER- β expression, some tumors and some cell lines were found to co-express these two genes. These data are consistent with previous findings (3,4) in which ER- β expression was found to have an over-lapping but non-identical tissue distribution to ER- α . While the radioactive PCR used to screen the breast tumor biopsy samples is a highly sensitive method likely to detect very low levels of expression of ER- β , several tumors presented a strong signal equivalent to that seen in the testis sample using both radioactive PCR and by ethidium bromide staining of PCR products. Our data suggest that ER- β may have a role in breast cancer cells, and this role may be expected to differ depending on the presence or absence of expression of the classical ER- α . Further, given the similarities and differences so far identified between these two gene products our results suggest that an involvement of ER- β in estrogen signal transduction or altered estrogen signal transduction in breast tissue will have to be considered.

Acknowledgments

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APPENDIX 11

Dotzlaw H, Leygue E, Watson PH, and Murphy LC

Estrogen receptor- β mRNA expression in human breast tumor biopsies:
relationship to steroid receptor status and regulation by progestins.

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Estrogen Receptor- β Messenger RNA Expression in Human Breast Tumor Biopsies: Relationship to Steroid Receptor Status and Regulation by Progestins¹

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Abstract

When the level of estrogen receptor (ER)- β mRNA in tumors, determined by reverse transcription-PCR, was assessed according to either ER status or PR status alone, determined by ligand binding assays, the level of ER- β mRNA was significantly lower in PR+ tumors compared with PR- tumors ($P = 0.036$), and no association with ER status was found. Subgroup analysis showed that ER- β mRNA expression in ER+/PR+ breast tumors was significantly less than in ER+/PR- ($P = 0.009$), ER-/PR+ ($P = 0.029$), and ER-/PR- ($P = 0.023$) groups. Interestingly, the ER- β mRNA expression was specifically decreased by progestin in T-47D breast cancer cells. The data suggest the possibility that expression of ER- β in human breast tumors is a marker of endocrine therapy responsiveness.

Introduction

Both estrogen and antiestrogen can mediate transcriptional activity via the recently identified ER- β (1-3). Recently, we have shown the presence of ER- β mRNA in both normal and neoplastic human breast tissues (4, 5). Furthermore, the relative expression of ER- α and ER- β mRNA changes between normal human breast tissues and their concurrent matched ER+ breast tumors (6), suggesting that altered expression of ER- α and ER- β occurs and may be functionally involved in breast tumorigenesis. Interestingly, it also seemed that the level of ER- β mRNA varied among breast tumors but was not correlated with the expression of ER- α (4), although the two receptor mRNAs were often coexpressed in the same tumor. These observations raised the question of whether the expression of ER- β in breast tumors was correlated with known prognostic and endocrine treatment response markers. In this study, the relationship of ER- β mRNA expression to ER and PR status, as determined by ligand binding analysis, was investigated.

Materials and Methods

Materials. All cell culture reagents were obtained from Life Technologies, Inc. (Burlington, Ontario). MPA and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). R5020 and Org 2058 were purchased from Amersham Corp. (Oakville, Canada). RU 486 was a gift from Roussel

Uclaf (Romainville, France). [α -³²P]dCTP was purchased from ICN (Montreal, Quebec).

Human Breast Tumors. Forty invasive ductal carcinomas were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected for ER and PR status as determined by ligand binding assays. Ten tumors were classified as ER+/PR+ (ER range, 50-127 fmol/mg protein; PR range, 105-285 fmol/mg protein); 10 tumors were classified as ER+/PR- (ER range, 59-156 fmol/mg protein; PR range, 5-10 fmol/mg protein); 10 tumors were ER-/PR- (ER range, 0-2 fmol/mg protein; PR range, 0-10 fmol/mg protein); and 10 tumors were classified as ER-/PR+ (ER range, 5-9 fmol/mg protein; PR range, 51-271 fmol/mg protein). These tumors spanned a wide range of grade (grade 4-9), determined using the Nottingham grading system.

Cell Culture. T-47D human breast cancer cells were obtained from Dr. D. Edwards (University of Colorado, Denver, CO). The cells were grown in DMEM supplemented with 5% fetal bovine serum, 100 nM glutamine, 0.3% (v/v) glucose, and penicillin/streptomycin, as previously described (7). Cells were plated at 1 times 10⁶ in 100-mm dishes and 2 days later were treated as indicated in the text. The steroids and other compounds were added directly from 1000 times stock solutions in ethanol to achieve the concentrations indicated. The cells were harvested at the times indicated by scraping with a rubber policeman. After centrifugation, the cell pellet was frozen and stored at -70°C until RNA was isolated.

RNA Extraction and RT-PCR Conditions. Total RNA was extracted from 20- μ m frozen tissue sections (5 sections/tumor) or frozen cell pellets using Trizol reagent (Life Technologies, Inc., Grand Island, NY), according to the manufacturer's instructions. Total RNA (1 μ g) was reverse transcribed in a final volume of 25 μ L, as previously described (4).

The primers used consisted of ER- β -U primer (5'-GTCCATCGCCAGT-TATCATC-3'; sense; located in ER- β 130-151) and ER- β -L primer (5'-GCCTTACATCCTTCACACGA-3'; antisense; located in ER- β 371-352). Nucleotide positions given correspond to published sequences of the human ER- β cDNA (2). PCR amplifications were performed, and PCR products were analyzed as previously described, with minor modifications (4). Briefly, 1 μ L of reverse transcription mixture was amplified in a final volume of 15 μ L, in the presence of 1.5 μ Ci [α -³²P]dCTP (3000 Ci/mmol), 4 ng/ μ L ER- β -U/ER- β -L, and 0.3 units of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. After electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed GAPDH cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide, as previously described (4). PCR products were subcloned and sequenced, as previously described (4).

Quantification and Statistical Analysis. Quantification of signals was carried out by excision of the band corresponding to ER- β cDNA, addition of scintillant, and scintillation counting. Three independent PCRs were performed. To control for variations between experiments, a value of 100% was assigned to the case exhibiting the highest signal measured, and all signals were expressed as a percentage of this signal. In parallel, GAPDH cDNA was amplified and, after analysis of PCR products on prestained agarose gels, signals were quantified by scanning using NIH Image 161/ppc software. Each GAPDH signal was also expressed as a percentage of the highest signal observed in the experiment. Two independent PCRs were performed. For each sample, the average of the ER- β signal was then expressed as a percentage of the average GAPDH signal. The statistical significance of any differences of

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³The abbreviations used are: ER, estrogen receptor; MPA, medroxyprogesterone acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PR, progesterone receptor; RT-PCR, reverse transcription-PCR.

the mean ER- β mRNA level between groups was determined using the Mann-Whitney test (two-tailed).

Results

Measurement of ER- β mRNA Expression in Primary Human Breast Tumors with Different ER and PR Status. Previous data have suggested that the level of ER- β mRNA varied widely in human breast tumor samples (4), which raised the question of whether the expression of ER- β in breast tumors was correlated with the known prognostic and treatment response variables, ER and PR status. Four groups, containing 10 breast tumor samples each, were identified according to their ER/PR status, as defined by ligand binding analysis (see "Materials and Methods"). ER- β mRNA levels were measured by RT-PCR and normalized to the GAPDH mRNA level, as measured in parallel by RT-PCR. The primers used in this analysis are located in exons 1 and 2 (Fig. 1A) of the human ER- β gene (2, 8) and would, therefore, measure the wild-type human ER- β mRNA and all ER- β mRNA variants so far documented (5, 9, 10). Examples of the results obtained are shown in Fig. 1B. The results obtained for all tumors assayed are shown in Fig. 1C, arranged in groups according to the ER/PR status of the tumor, as measured by ligand binding analysis.

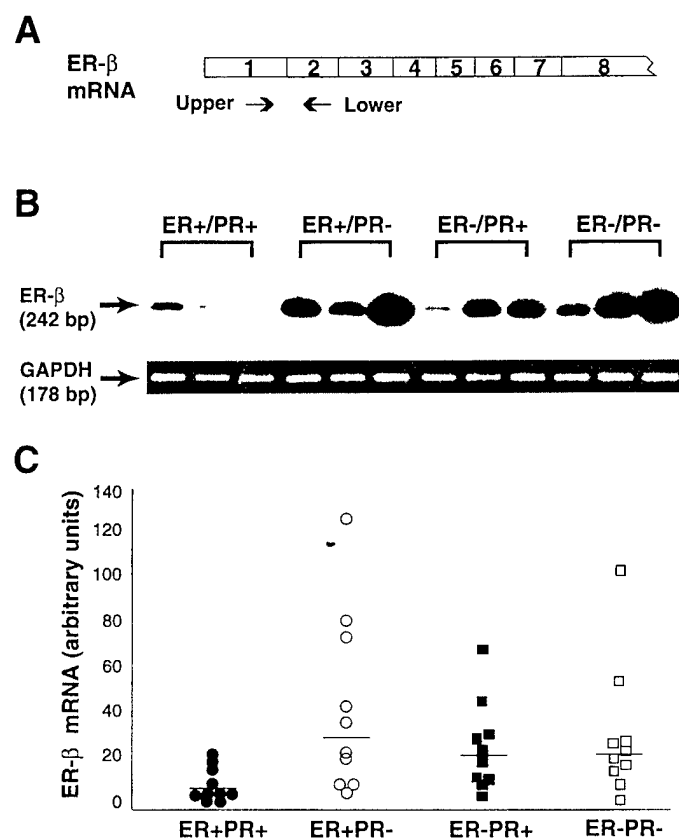


Fig. 1. A, schematic diagram of the human ER- β cDNA showing the priming sites of the upper and lower primers used for the analysis of ER- β mRNA by RT-PCR. B, expression of ER- β mRNA in human breast tumor biopsy samples, according to ER and PR status determined by ligand binding assay. Top, an autoradiogram of the RT-PCR assays for ER- β mRNA obtained from representative samples of tumors that were classified as ER+/PR+, ER+/PR-, ER-/PR+, and ER-/PR-, as described in "Materials and Methods." Bottom, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. C, quantification of ER- β mRNA expression within human breast tumors classified according to ER and PR status, as determined by ligand binding assay. Total RNA, extracted from the tumors, was reverse transcribed, PCR-amplified, and PCR products were separated on acrylamide gel as described in "Materials and Methods." Signals have been quantified and normalized, as indicated in "Materials and Methods." ●, ER+/PR+ tumors; ○, ER+/PR- tumors; ■, ER-/PR+ tumors; □, ER-/PR- tumors. Horizontal line, the median value in each group.

Table 1. Summary of ER- β mRNA levels according to steroid receptor status

ER/PR status	Number ^a	ER- β mRNA level (mean \pm SE)	Statistical significance ^b
A. ER+/PR+	10	11 \pm 7.2	
B. ER+/PR-	10	45 \pm 12	A vs. B, $P = 0.009$
C. ER-/PR+	10	26 \pm 6	A vs. C, $P = 0.029$
D. ER-/PR-	10	31 \pm 9.3	A vs. D, $P = 0.023$
E. ER+	20	28 \pm 7.2	
F. ER-	20	28 \pm 5.4	E vs. F, NS
G. PR+	20	19 \pm 3.5	
H. PR-	20	38 \pm 7.7	G vs. H, $P = 0.036$

^a Number of tumors/group.

^b Mann-Whitney test (two-tailed).

^c NS, not significant.

The level of ER- β mRNA in ER+/PR+ breast tumors was significantly less than in all other groups (see Table 1), with no significant differences seen among the ER+/PR-, ER-/PR+, or ER-/PR- groups. When the level of ER- β mRNA in tumors was assessed according to either ER status or PR status alone, as defined by ligand binding analysis, the level of ER- β mRNA was significantly lower in PR+ tumors compared with PR- tumors (Table 1, G versus H; $P = 0.036$), with no significant differences associated with ER status alone (Table 1, E versus F; $P = 0.323$).

Spearman correlation analysis showed no significant correlations of the level of ER- β mRNA with grade, age, nodal status, or the percentage of normal duct and lobular epithelium, stromal or fat cell content within the tissue section analyzed. However, an inverse relationship was found when the level of ER- β mRNA was correlated with the absolute level of PR, as measured by ligand binding analysis ($r = -0.31$; $P = 0.052$), consistent with the data when analyzed using clinically relevant cut-off values for both ER and PR status as shown above.

Regulation of Steady-state Levels of ER- β mRNA by Progestins in T-47D Human Breast Cancer Cells. The relationship of the level of ER- β mRNA with PR status in human breast tumor biopsies suggested the hypothesis that ER- β expression may be regulated by progestins. This hypothesis was investigated using the PR+ T-47D human breast cancer cell line in culture. The steady-state level of ER- β mRNA was found to decrease after treatment with 10 nM MPA (Fig. 2A). A significant decrease was observed at 6 hours after MPA treatment, and the levels remained decreased for up to 48 hours after treatment. The effect of MPA on the steady-state levels of ER- β mRNA in T-47D cells was first seen with 1 nM MPA and was maximal between 10 and 100 nM MPA (Fig. 2B). The progestin specificity of this response was assessed by treating T-47D cells for 24 hours with MPA, Org 2058, dexamethasone, and the antiprogestin RU 486 (Fig. 3, A and B). Both 10 nM MPA and 10 nM of the synthetic progestin Org 2058 significantly decreased the steady-state levels of ER- β mRNA, whereas little, if any, effect was observed with 100 nM of the synthetic glucocorticoid, dexamethasone. Antiprogestin/anti-glucocorticoid RU 486 (500 nM) had little, if any, effect by itself, but inhibited the down-regulation by 10 nM MPA on the level of ER- β mRNA. It was concluded that progestins can down-regulate the steady-state levels of ER- β mRNA and that an antiprogestin can inhibit this effect in T-47D human breast cancer cells.

Discussion

It was previously documented that the level of ER- β mRNA expression in human breast tumors varied widely (4, 8). This raised the question of whether the expression of ER- β in breast tumors was correlated with known prognostic and treatment-response markers. The measurement of both ERs and PRs in human breast biopsies is routinely used to provide both prognostic and treat-

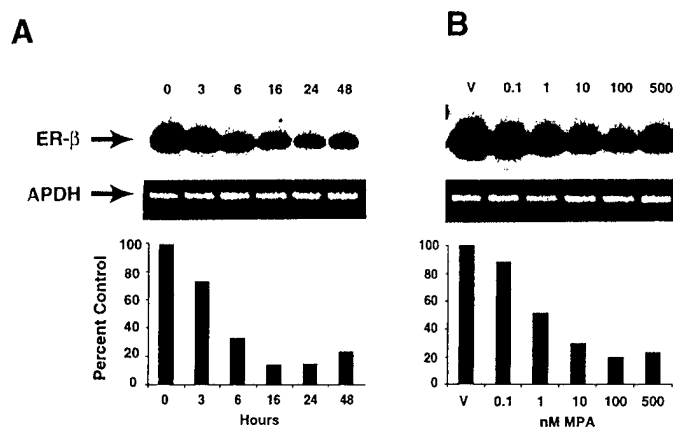


Fig. 2. A, time-dependent down-regulation of ER- β mRNA expression in T-47D human breast cancer cells. Top, an autoradiogram of ER- β mRNA levels determined by RT-PCR after treatment with 10 nM MPA for the indicated time periods. Middle, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. Bottom, the results presented as a histogram after quantification and normalization of the ER- β signal, as described in "Materials and Methods." This experiment was replicated twice. B, dose-dependent down-regulation of ER- β mRNA expression in T-47D human breast cancer cells. Top, an autoradiogram of ER- β mRNA levels determined by RT-PCR after treatment with vehicle alone (V) and varying concentrations of MPA for 24 h. Middle, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. Bottom, the results presented as a histogram after quantification and normalization of the ER- β signal, as described in "Materials and Methods."

ent-response information (11). Because ER- β is structurally and functionally related to ER- α (1–3, 12), it was relevant to determine whether the expression of ER- β was related to the ER and PR status of the tumor, as defined by ligand binding assays. Our analysis established that the expression of ER- β mRNA was inversely correlated with PR status generally. Although there was no significant correlation between ER- β mRNA levels and ER status overall, a significant difference in ER- β mRNA levels in those tumors that were ER+/PR+ (lowest expression) and those tumors that were ER-/PR+ (higher expression) was observed. This could be interpreted to mean that both ER status and PR status could influence ER- β mRNA expression. However, the differences observed could also be explained by the significant difference in the absolute level of PR expression between the two groups (PR levels determined by ligand binding assays expressed as mean \pm SE, 190 ± 24 fmol/mg protein versus 97 ± 21 fmol/mg protein, in ER+/PR+ and ER-/PR+ groups, respectively). This would be consistent with the inverse correlation that was seen with ER- β mRNA and the absolute levels of PR determined by ligand binding analysis, considering all groups together.

These data suggested the possibility that the expression of ER- β may be regulated by progestins. In T-47D cells (which express ER- α , ER- β , and PR), the steady-state level of ER- β mRNA was specifically decreased by progestin treatment in a time- and dose-dependent manner. Our data support the hypothesis that the progestin effect is mediated by PR, however, our data do not address whether this occurs via a transcriptional or post-transcriptional mechanism. Interestingly, progestins are known to also decrease the steady-state levels of ER- α mRNA and protein in T-47D cells (13). Therefore, PR is able to regulate the expression of both ER- α and ER- β in human breast cancer cells in a similar fashion. However, the interaction of PR and the two distinct ERs is likely to be different. It has been well documented that there is a general positive correlation between ER and PR levels, as determined by ligand binding assays in human breast tumors (11). ER status, as determined by ligand binding, correlates well with both immunological detection of the ER- α protein (14) and ER- α mRNA detection (15). Such data together with other studies

(6) suggest that the ER level in breast tumors, as determined by ligand binding in most cases, is due to ER- α . Furthermore, ER- β mRNA is the predominant ER mRNA in MDA MB 231 human breast cancer cells (4) and these cells are known to be ER negative by ligand binding assay providing further evidence for the lack of interference of ER- β expression in the determination of ER status by ligand binding assay in the majority of human breast tumors. Interestingly, a significant level of ER- β -like mRNA in human breast cancer cell lines and possibly, therefore, breast tumors may be represented by exon 8 deleted variants (10), which most likely encode nonestrogen binding ER- β variant proteins, which could not contribute to ER ligand binding assays. Therefore, the available data suggest that the previously observed positive correlation of ER and PR in human breast tumors is due to ER- α expression, underscoring the difference in the relationship of ER- α and ER- β with PR in human breast cancer tissue.

Our data are the first to identify a correlation between ER- β mRNA expression and a known prognostic and treatment-response marker in human breast cancer biopsies. The inverse relationship between PR (a good prognostic variable and a marker of response to endocrine therapies) and ER- β suggests that although ER- β is often down-regulated in human breast tumors compared with normal human breast tissue (6), its maintenance and/or increased expression in some breast tumors may correlate with a poorer prognosis and the likelihood of failure of response to endocrine therapies such as antiestrogens. This remains to be tested in samples of breast tumors from patients known to have responded or not to have responded to endocrine therapies, in clinical trials. Furthermore, a functional involvement of ER- β in this phenotype remains to be determined. Interestingly, although no agonist activity of tamoxifen-like antiestrogens can be measured through ER- β in a recombinant expression system using

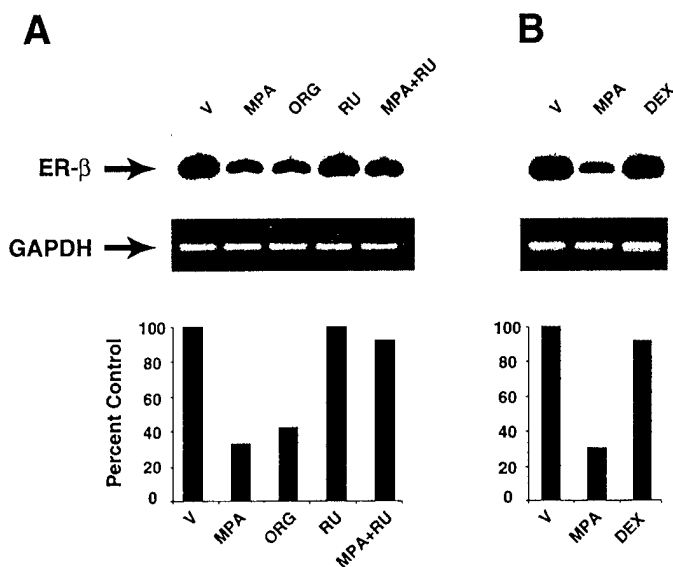


Fig. 3. A, steroid specificity of the down-regulation of ER- β mRNA expression in T-47D human breast cancer cells. Top, an autoradiogram of ER- β mRNA levels determined by RT-PCR after 24 h of treatment with vehicle alone (V), 10 nM MPA (MPA), 10 nM Org2058 (ORG), 500 nM RU 486 (RU), and 10 nM MPA + 500 nM RU 486 (MPA+RU). Middle, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. Bottom, the results presented as a histogram after quantification and normalization of the ER- β signal, as described in "Materials and Methods." This experiment was replicated twice. B, steroid specificity of the down-regulation of ER- β mRNA expression in T-47D human breast cancer cells. Top, an autoradiogram of ER- β mRNA levels determined by RT-PCR after 24 h of treatment with vehicle alone (V), 10 nM MPA (MPA), and 10 nM dexamethasone (DEX). Middle, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. Bottom, the results presented as a histogram after quantification and normalization of the ER- β signal, as described in "Materials and Methods."

transient transfection and a classical ERE-reporter gene (3), all classes of antiestrogens bound to ER- β result in the transcriptional activation of AP-1-driven reporter genes, again in a transient recombinant model system (12). Because AP-1-regulated genes are often associated with growth and proliferation (16–18), it is tempting to speculate that increased expression of ER- β in human breast tumors could play a role in tamoxifen resistance in the small number of tumors that appear to proliferate in response to tamoxifen (19, 20).

Acknowledgments

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APPENDIX 12

Leygue E, Dotzlaw H, Hare, H., Watson PH, and Murphy LC

Expression of estrogen receptor-beta (ER- β) variant mRNAs
in human breast tumors.

20th Annual Breast Cancer Symposium.
San Antonio, Texas, Abstract 175, 1997.

- 173 TAMOXIFEN DOWN REGULATES PHORBOL 12-MYRISTATE 13-ACETATE INDUCED NITRIC OXIDE PRODUCTION IN ZR-75-1 HUMAN BREAST CANCER CELLS. Alalami O* & Martin JHJ Division of Biomedical Sciences, School of Health Sciences, University of Wolverhampton, Wolverhampton, England, WV1 1DJ.

The purpose of this study was to determine if the L-arginine/NO pathway was present in ZR-75-1 human breast cancer cells and to investigate possible effects of tamoxifen.

ZR-75-1 cells (5×10^4 cells/ml) were cultured in the presence and absence of various drugs. After 2 days *in vitro* the NO_2^- concentration in conditioned medium was quantitated by the Greiss reaction.

Treatment of ZR-75-1 cells with L-arginine (10mM) increased nitrite production from 2nmol to 70nmol ($p < 0.001$). This could be significantly ($p < 0.001$) inhibited by L-NAME (2mM). Treatment with phorbol 12-myristate 13-acetate (PMA) (200nM-1000nM) caused a significant increase ($p < 0.001$) in NO_2^- secreted into the culture medium. Although tamoxifen (10^{-8} M) had no effect on control levels of NO_2^- production, tamoxifen was able to significantly ($p < 0.001$) down-regulate PMA enhanced nitrite production by ZR-75-1 cells.

We conclude that tamoxifen can down regulate PMA induced NO production by ZR-75-1 human breast cancer cells. As nitric oxide has been implicated in several areas of tumour biology including metastasis, differentiation and angiogenesis we suggest that further investigations of the effects of anti-estrogens on this pathway would be of value.

- 174 CORRELATION BETWEEN GCDFF-15 AND HORMONE RECEPTORS IN HUMAN BREAST CARCINOMA. Wheeler HJ*, Moe RE, Gown AM, University of Washington Medical Center, Seattle, Washington 98195.

Breast carcinomas are known to be influenced by hormones, particularly estrogens and progestins. Androgen manipulation is used much less often to treat breast cancer. On the other hand, some breast cancers not only respond to androgens but progress instead of regressing with androgen therapy. A glycoprotein in breast cyst fluid, gross cystic disease fluid protein (GCDFF-15), appears in these cancers and in the peripheral blood of patients with these androgen-sensitive cancers.

This study tests the hypothesis that GCDFF-15 expression correlates with androgen receptor expression, but does not correlate with non-androgen hormone receptor expression. Correlation between expression of GCDFF-15, androgen, estrogen, and progesterone receptors was evaluated. Immunocytochemistry studies were performed on a series of 50 human breast tissue specimens, 48 with breast carcinomas and two with benign breast disease. Specimens were scored in a semi-quantitative fashion based on distribution of positive cells within the breast tumor or benign tissue, followed by a chi-square statistical analysis.

A strong correlation was found between GCDFF-15 expression and androgen receptor expression in these breast cancers ($P = 0.02$). Estrogen receptor expression in these cancer specimens was correlated with androgen receptor expression ($P = 0.002$), but the former was not significantly associated with GCDFF-15 expression ($P = 0.35$). These results support the finding of a unique subset of breast cancers correlated with GCDFF-15 and potentially stimulated by androgens. In women with cancers positive for GCDFF-15 and androgen receptors, androgen antagonists such as Flutamide might be beneficial.

- 175 EXPRESSION OF ESTROGEN RECEPTOR-BETA VARIANT mRNAs IN HUMAN BREAST TUMORS.

Leygue E*, Dotzlaw H, Hare H., Watson PH*, Murphy LC. Department of Biochemistry and Molecular Biology and Department of Pathology*, University of Manitoba, Winnipeg, Canada, R3E0W3.

The presence of estrogen receptor-beta (ER- β) mRNA within human breast tumors and breast cancer cell lines has recently been reported (Dotzlaw et al. J Clin Endocrinol Metabolism, in press). Because several exon-deleted variant forms of the classic estrogen receptor (ER- α) have been described and are suspected to be involved in the acquisition of the hormone-independent phenotype during breast tumorigenesis, it was our aim to determine whether ER- β variant mRNAs might be expressed in human breast tumor specimens. To address this issue, a recently described RT-PCR approach (Cancer Res. 56:4324, 1996), based on the co-amplification of wild-type ER- β and all possible variant mRNAs containing putative exon 1 and exon 8 of the receptor, whose precise exonic structure is not yet known, has been used. Several PCR products were obtained in both breast tumor samples and breast cancer cell lines. The sequence of one of these bands, co-amplified with wild-type ER- β in several tumor samples and breast cancer cell lines, revealed a deletion of 273 bases that by analogy to the ER- α exonic structure, would correspond to the deletion of putative exons 5 and 6. This deleted ER- β variant mRNA, if translated, would encode an ER- β like protein truncated in 91 amino acids within the region containing the hormone binding domain and the transactivating function 2 (AF-2) of the ER- β protein. These results suggest that ER- β variants exist, and as suggested for ER- α variants might also be involved in mechanisms underlying tumor progression.

- 176 DISSOCIATION BETWEEN STEROID RECEPTOR EXPRESSION AND CELL PROLIFERATION IN THE NORMAL HUMAN MAMMARY GLAND.

Clarke RB*, Howell A* and Anderson E. Clinical Research Department and *CRC Department of Medical Oncology, (University of Manchester), Christie Hospital (NHS) Trust, Wilmslow Road, Manchester, M20 4BX, UK.

Oestradiol (E_2) stimulates cell proliferation and progesterone receptor (PgR) synthesis in luminal epithelial cells of the normal breast, presumably acting via the specific nuclear E_2 receptor (ER). Approximately 10% of epithelial cells within the breast express immuno-detectable ER but little is known about their distribution and their organisation in relationship to proliferating cells and those expressing the PgR. We performed double labelling on breast tissue sections by an indirect peroxidase method using antibodies to ER or PgR and ^3H -thymidine (^3H -dT) histautoradiography, and also by immuno-fluorescence using antibodies to steroid receptors and the Ki67 proliferation antigen. The results from normal human breast tissue indicate that ER-positive cells are distributed evenly throughout the mammary epithelium. These cells also synthesise the PgR but are rarely observed to divide (see table). Conversely, in ER-positive human mammary tumours, a significantly higher proportion of dividing cells express ER (22.4%; range 0-84%). These data indicate that normal breast epithelial cells capable of proliferation do not express ER and may be stimulated by E_2 indirectly via secretion of paracrine factors. In some tumours, however, cells may have acquired proliferative capacity and be directly stimulated by E_2 .

	Number of Samples	Total Cells	ER+ve (%)	PgR+ve (%)	^3H -dT (%)	Ki67 (%)	Receptor+ve and Proliferative (%)
Normal	10	10,026	1735 (17.3)	-	46 (0.5)	-	1 (2)
Normal	25	25,302	-	3232 (12.8)	382 (1.5)	-	17 (4.7)
Normal	25	28,395	2107 (7.4)	-	-	639 (2.3)	9 (1.3)
Normal	25	28,018	-	3231 (11.5)	-	391 (1.4)	7 (1.4)
Normal	13	13,895	1792 (12.9)	1765 (12.7)	-	-	1727 (96*)
Tumour	19	21,245	6693 (31.5)	-	-	1235 (5.8)	275 (22.4)

† Laidlaw et al. (1995) Endocrinol. 136, 164-171.

*Receptors coexpressed

APPENDIX 13

Lu B, **Leygue E**, Dotzlaw H, Murphy LJ, Murphy LC, and Watson PH

Estrogen receptor beta mRNA variants in human and murine tissues.

Mol Cell Endocrinol, 138:199-203, 1998.

Rapid communication

Estrogen receptor- β mRNA variants in human and murine tissues

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Abstract

Estrogen receptor (ER)- β mRNA splice variants have been identified in human breast tumors as well as normal human and mouse ovarian, uterine and mammary tissues. In both species transcripts deleted in exons 5 or 6, or 5 + 6 have been characterized by RT-PCR followed by cloning and sequencing. In mouse tissues an ER- β transcript containing 54 nucleotides inserted in frame between exons 5 and 6 was identified. Interestingly, no equivalent of the mouse inserted transcript was detected in any of the four human tissues analyzed. © 1998 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Estrogen receptor- α ; Estrogen receptor- β ; mRNA variant; Alternative splicing; Human; Mouse

1. Introduction

Recently, the cDNA of a second estrogen receptor, estrogen receptor (ER)- β , was cloned and sequenced from the rat (Kuiper et al., 1996), the human (Mosselman et al., 1996) and the mouse (Tremblay et al., 1997). Northern analysis of RNA isolated from mouse ovary demonstrated the presence of multiple mRNA species for ER- β (Tremblay et al., 1997) suggesting the possibility that variant ER- β proteins might exist. To investigate whether ER- β variant mRNAs might be expressed in human as well as murine tissues, an RT-PCR analysis was undertaken which demonstrated the presence of variant ER- β mRNAs in both species.

2. Materials and methods

2.1. Tissues and RNA extraction

Human breast tumor specimens left-over from steroid receptor assays were obtained from the Manitoba Breast Tumor Bank, and three non-malignant human uterine hysterectomy samples were obtained from the Department of Obstetrics and Gynecology (Health Sciences Centre, Winnipeg, Canada). Total RNA was extracted by the guanidinium thiocyanate/cesium chloride method as previously described (Dotzlaw et al., 1990). Four nonmalignant human ovarian samples from two pre-menopausal and two post-menopausal women were obtained through the Ovarian Tissue Bank (Institut du Cancer de Montreal, Centre de Recherche Louis-Charles Simard, Montreal, Canada). Four normal human breast tissues from reduction mammoplasties of pre-menopausal women were obtained through the Manitoba Breast Tumor Bank. Total RNA from the ovarian and normal

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breast tissue samples was extracted with Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

Mouse uteri and ovaries were obtained from four female mice aged 8–9 weeks, and mammary tissues were obtained from two adult lactating female mice. Total RNA was extracted with Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

Integrity of RNA was confirmed by denaturing gel electrophoresis as previously described (Murphy and Dotzlaw, 1989).

2.2. RT-PCR and primers

Total RNA (1.5 μ g per reaction) was reverse transcribed as previously described (Dotzlaw et al., 1997). One microlitre of this reaction was amplified by PCR incorporating 32 P in a final volume of 10 μ l, and 4 μ l of this reaction separated on 6% denaturing polyacrylamide gels and autoradiographed as previously described (Dotzlaw et al., 1997).

All ER- β exons are defined in this report by analogy to the human ER- β exon structure (Enmark et al., 1997): human primer set one: hER- β exons 4 and 7: hER- β -4 (sense) 5'-GGC CGA CAA GGA GTT GGT A-3' (priming site in exon 4, nucleotides 762–780 as numbered in Mosselman et al. (1996)); hER- β -7 (antisense) 5'-TCC ATG CCC TTG TTA CTC G-3' (priming site in exon 7, nucleotides 1262–1280). The PCR conditions were 30 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C.

Human primer set two: hER- β exons 5 and 6: hER- β -5 (sense) 5'-GCT GTT GGA TGG AGG TGT TA-3' (priming site in exon 5, nucleotides 857–876); hER- β -6 (antisense) 5'-CTT GAA GTA GTT GCC AGG AG-3' (priming site in exon 6, nucleotides 997–1016). The PCR conditions were 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C.

Mouse primer set one: mER- β exons 4 and 8: mER- β -4 (sense) 5'-CTG AAC AAA GCC AAG AGA-3' (priming site in exon 4, nucleotides 600–617 as numbered in Tremblay et al. (1997)); mER- β -8 (antisense) 5'-GCT CTT ACT GTC CTC TGT CG-3' (priming site in exon 8, nucleotides 1417–1436). The PCR conditions were 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C.

Mouse primer set two: mER- β exons 5 and 6: mER- β -5 (sense) 5'-GCT GAT GGT GGG GCT GAT GT-3' (priming site in exon 5, nucleotides 890–909); mER- β -6 (antisense) 5'-ATG CCA AAG ATT TCC AGA AT-3' (priming site in exon 6, nucleotides 993–1012). The PCR conditions were 35 cycles of 1 min at 94°C, and 30 s at 60°C.

PCR products from human breast tumors and mouse mammary tissues were subcloned into the cloning vector, pGEM-T Easy (Promega) following the manufac-

turer's instructions. Double stranded DNA from at least two independent clones from each tissue was sequenced with a T7 Sequencing kit (Pharmacia) following the manufacturer's protocol. All RT-PCRs were carried out at least 2 \times for each sample analyzed.

3. Results and discussion

Previously the presence of ER- β mRNA was identified in some human breast tumor samples (Dotzlaw et al., 1997). The RT-PCR analysis employed a primer set which annealed to sequences corresponding to exons 7 and 8 of the human ER- β cDNA (Enmark et al., 1997). Numerous splicing variants of the human ER- α mRNA have been identified to date (Murphy et al., 1997), and it was of interest to determine if similar splice variants could be detected in the ligand binding domain of the ER- β mRNA in human breast tumors. Using a primer set which would anneal to sequences located in exons 4 and 7 of the human ER- β cDNA (Enmark et al., 1997), RT-PCR analyses were undertaken using RNA isolated from four separate human breast tumor samples which had previously been shown to express ER- β mRNA by RT-PCR using an exon 7/8 primer set (Dotzlaw et al., 1997). The results presented in Fig. 1 show the presence of the expected 519 bp wild-type ER- β product, as well as several smaller sized PCR products.

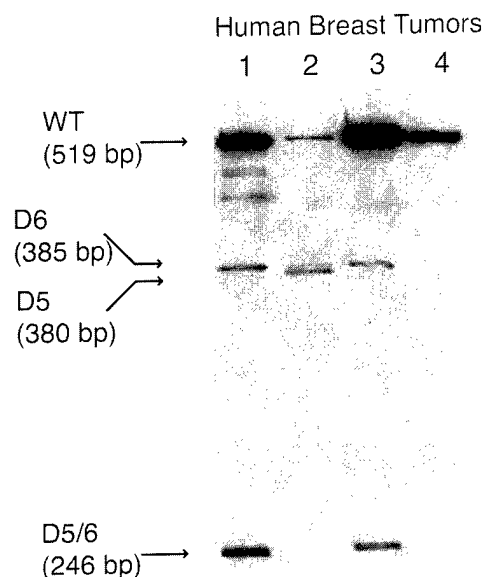


Fig. 1. Detection of wild-type ER- β and ER- β variant mRNAs in human breast tumor tissues. Total RNA extracted from four different human breast tumors (1–4) was reverse transcribed and PCR amplification was carried out using primers located in exons 4 and 7. PCR products migrating at the sizes of 519, 385, 380 and 246 bp were subsequently cloned, sequenced and identified as corresponding to ER- β wild-type (WT), exon 6-deleted variant (D6), exon 5-deleted variant (D5) and exon 5–6-deleted variant (D5/6) cDNAs, respectively.

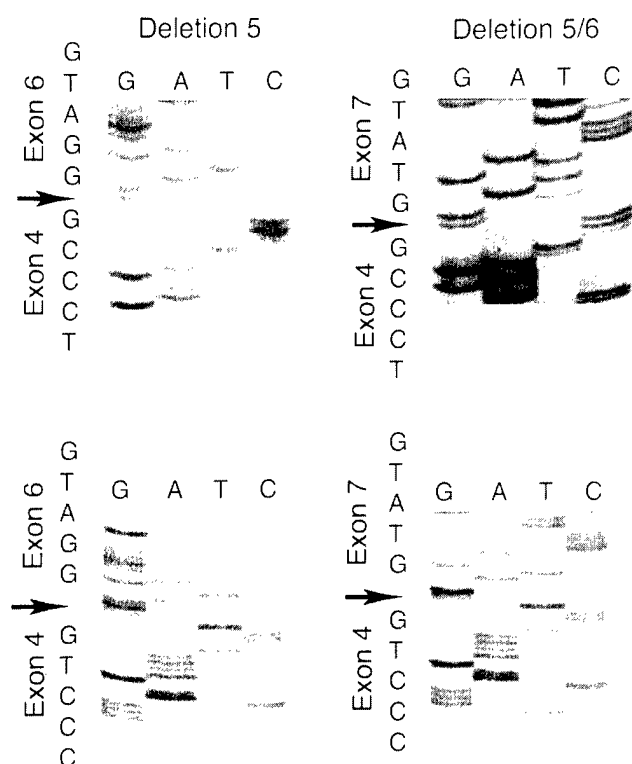


Fig. 2. Sequencing of exon 5-deleted and exon 5/6-deleted ER- β variants in human and murine tissues. Top panels: sequencing of PCR products obtained by amplification of human breast tumor cDNAs using primers in exons 4 and 7 and migrating at the sizes of 380 and 246 bp (Fig. 1) showed a perfect junction between exon 4 and 6 (deletion 5), and exon 4 and 7 (deletion 5/6), respectively. Bottom panels: sequencing of PCR products obtained by amplification of murine breast tissue cDNAs using primers in exons 4 and 8 and migrating at the sizes of 698 and 564 bp (Fig. 3) showed a perfect junction between exon 4 and 6 (deletion 5), and exon 4 and 7 (deletion 5/6), respectively.

Cloning and sequencing of the smaller sized products (Fig. 2) revealed deletions of nucleotides 812–950, 951–1084 and 812–1084 (numbered as in Mosselman et al. (1996)) which are precise exon deletions of exon 5, 6, and 5 + 6, respectively. To determine if such deletions occurred only in human breast tumor tissue, RNA extracted from several normal breast, uterine and ovarian tissue samples was analyzed (Fig. 3, top panel). The same tissues from the mouse were analyzed in parallel, with mouse ER- β primer set located in putative exons 4 and 8 (Fig. 3, bottom panel; all exons, mouse and human, are numbered according to the human ER- β structure (Enmark et al., 1997)). All human tissues analyzed expressed ER- β variant mRNAs similar to those identified in breast tumors. The expected wild-type product of 837 bp was detected in all mouse tissues, as were several smaller sized PCR products. Cloning and sequencing of the 698 and 564 bp fragments identified deletions of nucleotides 829–967 and 829–1101, which by analogy to the human ER- β would be a precise deletion of exon 5 and deletion of exons 5 and 6, respectively (Fig. 2, nucleotides numbered as in

Tremblay et al. (1997)). The 703 bp band was found to correspond to an exon 6 deleted ER- β transcript.

These data are the first to support the expression of exon deleted splice variants for ER- β similar to those for ER- α , in both human and murine tissue samples. The exon 5 and 6 deleted splice variants identified in this

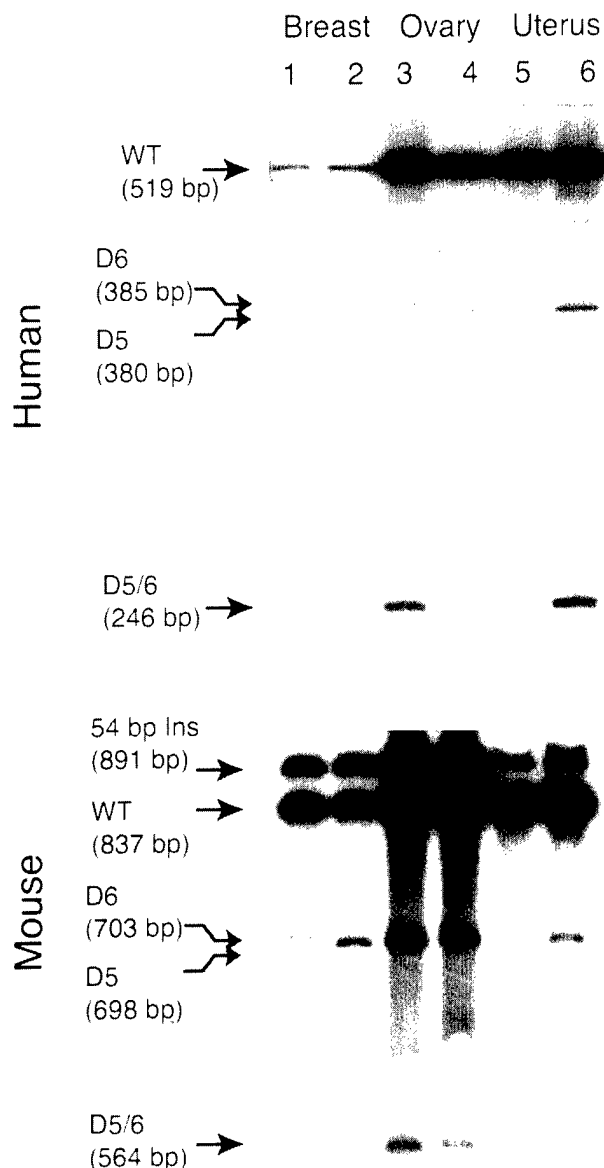


Fig. 3. Detection of wild-type ER- β and ER- β variant mRNAs in normal human and murine tissues. Total RNA extracted from normal human (top panel) or mouse (bottom panel) breast tissues (1–2), ovaries (3–4) and uteri (5–6) was reverse transcribed and PCR amplification was carried out using primers located in exons 4 and 7 (human) or in exons 4 and 8 (mouse). PCR products obtained in human tissues migrated at the sizes of 519, 385, 380 and 246 bp corresponding to ER- β wild-type (WT), exon 6-deleted (D6), exon 5-deleted (D5) and exon 5–6-deleted (D5/6) cDNAs, respectively. PCR products obtained in mouse tissues and migrating at the sizes of 891, 837, 703, 698 and 564 bp were identified as corresponding to a 54 bp inserted ER- β variant (54 bp Ins), ER- β wild-type (WT), exon 6-deleted variant (D6), exon 5-deleted variant (D5) and exon 5–6-deleted variant (D5/6), respectively.

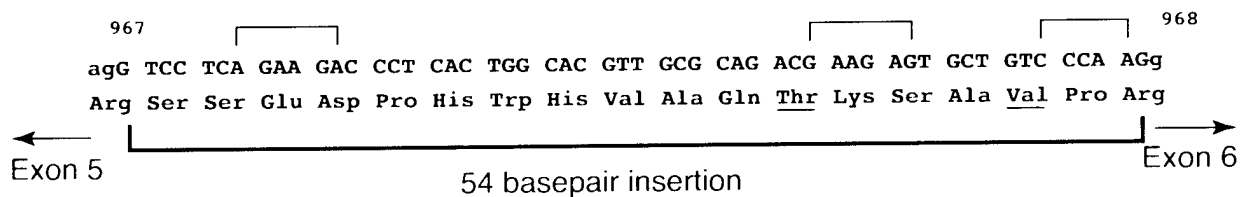


Fig. 4. Insertion (54 bp): nucleotide and amino acid sequences. The sequence of the inserted 54 bp is indicated in upper case letters. Nucleotides corresponding to the published mouse ER- β cDNA sequence, indicated in lower case letter, are numbered according to Dotzlaw et al. (1990). Brackets indicate putative exonic splicing enhancer sequences (Coulter et al., 1997; Otto et al., 1997). Predicted amino acid composition of the insert is shown. The underlined amino acids correspond to the substitutions observed between mouse inserted sequence and the recently described rat inserted sequence (Daffada et al., 1994).

study are out-of-frame and would be expected to encode C-terminally truncated ER- β proteins, which are unlikely to bind ligand. The exon 5 + 6 deleted splice variant is inframe but deleted in 91 amino acids (aa) (274–364 of the mouse and human ER- β (Enmark et al., 1997)), which are within the hormone-binding domain. The putative protein encoded by the exon 5 + 6 deleted variant would also be unlikely to bind ligand.

In contrast to what was observed in human tissues, all murine tissues analyzed presented a prominent ER- β 891 bp PCR product which was larger than the expected wild-type ER- β product of 837 bp (Fig. 3, bottom panel). Sequencing of the larger PCR product revealed an insertion of 54 nucleotides between nucleotides 967 and 968 (Tremblay et al., 1997), which is precisely inserted between the splice junction of exons 5 and 6. Identical results were obtained when the starting RNA samples were enriched for polyadenylated transcripts using oligo-dT attached to magnetic beads (data not shown), suggesting that the inserted transcript represented an authentic mRNA species. The sequence of this insertion is shown in Fig. 4. This insertion is inframe and the predicted amino acids are shown in Fig. 4 also. While this work was in progress the presence of a 54 bp inserted ER- β transcript in rat tissues was published (Chu and Fuller, 1997). The sequence of the 54 nucleotide insertion in the murine ER- β transcript is identical to that published for the rat except for a T \rightarrow C change at nucleotide position 36 (1 = start of the 54 nucleotide insert), which would result in a Met \rightarrow Thr substitution in the mouse protein, and a C \rightarrow T change at position 48 which would result in an Ala \rightarrow Val substitution in the mouse protein.

Because the initial screening of human tissues using the exon 4/7 primer set failed to reveal an analogous human ER- β transcript containing an insertion between exons 5 and 6, reanalysis of human and mouse tissues was undertaken using primer sets located in exons 5 and 6 of either the human or the mouse ER- β . While the inserted ER- β transcript was easily detected as a 177 bp PCR product in all murine tissues analyzed, only the expected 160 bp PCR product corresponding to the human wild-type ER- β mRNA was detected in the normal human tissues (Fig. 5), and an inserted ER- β variant was similarly not detected in ten human ER- β RNA positive

breast tumor samples (Dotzlaw et al., 1997) (data not shown). The data shown in Fig. 5 suggest that the inserted transcript is predominant in both mouse mammary gland and uterus, while similar levels of each transcript occur in the mouse ovary. It should be noted that the primers used in this latter analysis (Fig. 5) were designed to detect the mouse ER- β wild-type and the inserted transcript. Thus only two competing PCR products are obtained, and measurement of relative expression using such an approach has been validated previously (Daffada et al., 1994; Leygue et al., 1996). This is in contrast to the primer set used in Fig. 3 which detects up to five PCR products, is designed for the study of relative patterns of expression and is unlikely to accurately reflect the relative expression of any two individual species under such conditions.

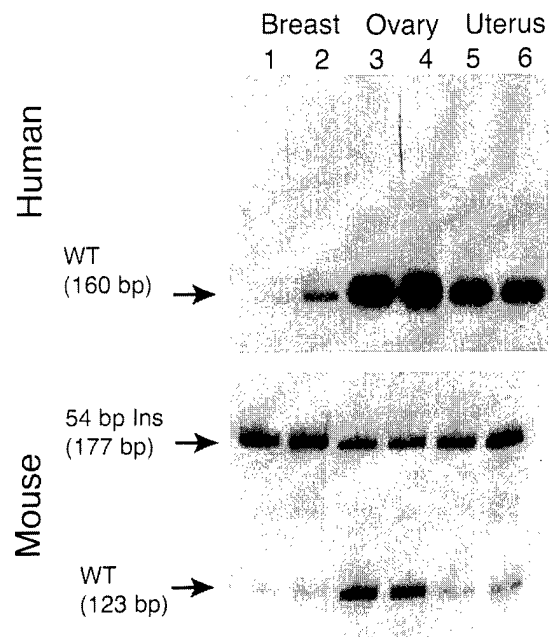


Fig. 5. Amplification of human and murine cDNAs using exon 5 and 6 primers. Total RNA extracted from normal human (top panel) or mouse (bottom panel) breast tissues (1–2), ovaries (3–4) and uteri (5–6) was reverse transcribed and PCR amplification was carried out using primers located in exons 5 and 6. The PCR product obtained in human tissues migrated at the size of 160 bp corresponding to ER- β wild-type (WT). PCR products obtained in mouse tissues migrated at the sizes of 177 and 123 bp, corresponding to a 54 bp inserted ER- β variant (54 bp Ins) and ER- β wild-type (WT), respectively.

A possible mechanism associated with the frequent inclusion of the inserted sequences in mouse ER- β transcripts may be the presence of several putative exonic splicing enhancer sequences within the insertion sequences (Cooper and Mattox, 1997; Coulter et al., 1997). Both purine rich motifs and A/C-rich splicing enhancer sequences are present (brackets in Fig. 4). The frequent inclusion of the inserted sequences in mouse ER- β transcripts, and their presence in several tissues at comparable levels with the wild-type transcript suggest that the protein encoded by the inserted transcript has a functional role, at least in the mouse and rat (Chu and Fuller, 1997). The putative function of a protein encoded by the inserted mouse ER- β transcript is unknown, and since the insertion is in the middle of the ligand binding domain, it may either disrupt binding completely or result in a different ligand binding specificity and/or affinity. Further, the insertion may effect the three dimensional structure of the ER-domain such that alterations in dimerization, transactivation and interaction with co-regulators may also occur. This could result in the inserted ER- β having a regulatory function on the wild-type ER- β as previously suggested (Chu and Fuller, 1997), or may completely alter its ability to heterodimerize and affect the activity of ER- α (Cowley et al., 1997). The lack of detection of a similar inserted ER- β transcript in human tissues may be due to hormonal differences at the time of tissue collection between the mouse and human subjects, or to a real species difference in alternative splicing. In the latter instance differences in alternative splicing between mouse and human with regard to a steroid hormone receptor have been previously documented (Oakley et al., 1996; Otto et al., 1997). Similarly, inserted sequences within the ligand binding domain of ER- α have also been reported (Murphy et al., 1996). In contrast with the inserted ER- β mRNA, the inserted ER- α mRNA was detected in one human breast tumor sample, and was due to a point mutation in one allele of the human ER- α gene present in the breast tumor (Wang et al., 1997).

In summary, in this report deletion splice variants of ER- β have been characterized for the first time in several mouse and human tissues, both normal and neoplastic. An inserted splice variant of the ER- β mRNA, previously identified in the rat, has been confirmed in several mouse tissues. This inserted variant was undetected in any human tissues analyzed, suggesting species specific differences in its expression.

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(MRC) Scientist, P.H.W. is a MRC clinician-scientist, E.L. is a recipient of a USAMRMC Postdoctoral Fellowship. The authors thank Heidi Hare for excellent technical assistance.

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APPENDIX 14

Lu B, Dotzlaw H, **Leygue E**, Murphy LJ, Watson PH, and Murphy LC

Estrogen receptor alpha mRNA variants in murine and human tissues.

Mol Cell Endocrinol, In press.

**ESTROGEN RECEPTOR- α mRNA VARIANTS IN MURINE AND
HUMAN TISSUES.**

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Abbreviated Title: Species Differences in ER α Variant mRNAs.

Keywords: estrogen receptor- α , variant mRNAs, species differences, alternative splicing.

SUMMARY.

A side-by-side comparison of several normal mouse and human tissues was undertaken in order to determine if exon deleted variant ER- α mRNAs are expressed in the mouse. The data showed that the complex pattern of ER- α alternative splicing that is detected in multiple human tissues was not apparent in murine tissues. Only low levels of an exon 4 deleted ER- α transcript were detected in murine tissues, although multiple relatively abundant exon deleted ER- α transcripts were detected in human tissues. The data support a species specific difference in the expression of ER- α variant mRNAs between mouse and human.

INTRODUCTION.

A large body of data support the expression of multiple splice variants of the estrogen receptor- α (ER) mRNA in both normal and neoplastic human tissues [1,2]. The most prevalent pattern of variant ER transcript is the precise deletion of one or more exons [3]. In contrast the expression of similar exon deleted ER variant transcripts in rodent tissues has not been investigated in detail. The detection of an exon 4 deleted ER mRNA species following two rounds of PCR amplification was reported using polyA⁺ RNA isolated from hypothalamic-enriched rat brain tissue and rat uterine tissue [4]. However, it was noted in this latter study that only trace amounts of the variant ER were detected in rat uterine tissue when compared to the brain tissue. Exon 4 deleted, exon 4+5 deleted and exon 3+4 deleted ER transcripts were detected in rat aortic smooth muscle and bone cells using a similar approach of reverse transcription followed by two rounds of PCR amplification [5,6]. From these studies in which there was the need to use two rounds of PCR amplification for detection, it seemed that expression of ER variants in rodent tissues was less frequent and less abundant than in human tissues. However, the majority of studies in the human have been done in breast cancer tissues and cells, and there is some evidence to suggest that variant mRNA expression is downregulated in normal human breast tissues compared to breast tumors [7,8]. Although this is not the case for all ER variant mRNAs [9]. However, it was possible that the perception of a higher frequency of detection of variant ER mRNA expression in human versus rodent tissue might be due to the more frequent study of cancerous human tissues. To our knowledge there has been no side-by-side analysis of human and rodent ER variant expression. So to address the question of variant ER mRNA expression in rodent and human

tissues we have undertaken a side-by-side comparison of several non-neoplastic murine and human tissues.

MATERIALS AND METHODS.

Tissues and RNA extraction.

Non-malignant human uterine hysterectomy samples were obtained from the Department of Obstetrics and Gynecology, Health Sciences Centre, Winnipeg, Canada. Total RNA was extracted using the guanidinium thiocyanate/cesium chloride method as previously described [10]. Non-malignant human ovarian samples were obtained through Dr Mes-Masson and the Ovarian Tissue Bank at the Institut du Cancer de Montreal, Centre de Recherche Louis-Charles Simard, Montreal, Canada. Normal human breast tissues from reduction mammoplasties of pre-menopausal women were obtained through the Manitoba Breast Tumor Bank. Total RNA from the ovarian and normal breast tissue samples was extracted using Trizol reagent (Gibco/BRL) according to the manufacturer's instructions, and the integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described [11].

Murine tissues except for mammary tissues were obtained from female mice aged 8 to 9 weeks, while mammary tissues were obtained from two adult lactating female mice. Murine studies were conducted in accordance with the principles and procedures recommended by the University of Manitoba Animal Care Review Board. Total RNA was extracted using Trizol reagent (Gibco/BRL) according to the manufacturer's instructions, and the integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described [11].

RT-PCR and Primers.

Total RNA (1.5 µg per reaction) was reverse transcribed as previously described [12]. One µl of this reaction was amplified by PCR incorporating ^{32}P in a final volume of 10 µl, and 4 µl of this reaction separated on 6% denaturing polyacrylamide gels and autoradiographed as previously described [12]. Routinely PCR was carried out for 35 cycles, where one cycle consisted of 1 min 94°C (denaturation), 1 min 60°C (annealing) and 2 min 72°C (extension).

To avoid confusion with respect to the numbering of exons and to facilitate comparisons of the human and murine ER- α primer sets, all mER- α exons are defined in this report by analogy to the human ER- α exon/intron structure (see Figure 1). Primer design was facilitated by the use of the software programs OLIGO (version 4.0s, National Biosciences Inc, Plymouth, MN) and Amplify (version 1.2, Genetics, U of Wisconsin, Madison, WI):

Mouse ER- α exon 1 and 8 primer set : mER 1-8 (sense) 5' - GCC GCC TTC AGT GCC AAC AG - 3' (priming site in exon 1, nucleotides 461-480 as numbered in reference [13]); mER 1-8 (antisense) 5' - AGG AAT GTG CTG AAG TGG AG - 3' (priming site in exon 8, nucleotides 1918-1937 [13]).

Mouse ER- α exon 2 and 5 primer set : mER 2-5 (sense) 5'- GCA GTA ACG AGA AAG GAA AC - 3' (priming site in exon 2, nucleotides 702-721 as numbered in reference 13); mER 2-5 (antisense) 5' - CGA GAC CAA TCA TCA GAA TC - 3' (priming site in exon 5, nucleotides 1357-1376 [13]).

Mouse ER- α exon 4 and 7 primer set: mER 4-7 (sense) 5' - ATC AAC TGG GCA AAG AGA GT - 3' (priming site in exon 4, nucleotides 1275-1293 [13]); mER 4-7 (antisense) 5' -TCA AGG TGC TGG ACA GAA AC - 3' (priming site in exon 7, nucleotides 1582-1601 [13]).

Mouse ER- α exon 6 and 8 primer set: mER 6-8 (sense) 5' - AGG GTG AAG AGT TTG TGT GC - 3' (priming site in exon 6, nucleotides 1524-1543 [13]); mER 6-8

(antisense) 5' - AGG AAT GTG CTG AAG TGG AG - 3' (priming site in exon 8, nucleotides 1918-1937 [13]).

Human ER- α exon 1 and 8* primer set: hER- α 1-8* (sense) 5' - TGC CCT ACT ACC TGG AGA ACG - 3' (priming site in exon 1, nucleotides 615-637 [14]); hER- α 1-8* (antisense) 5' - GCC TCC CCC GTG ATG TAA - 3' (priming site in exon 8, nucleotides 1995-1978 [14]).

Human ER- α exon 2 and 6 primer set: hER- α 2-6 (sense) 5' - AGG GTG GCA GAG AAA GAT - 3' (priming site in exon 2, nucleotides 708-725 as numbered in reference [14]); hER- α 2-6 (antisense) 5' - ATG CGG AAC CGA GAT GAT GTA GC - 3' (priming site in exon 6, nucleotides 1520-1542 [14]).

Human ER- α exon 4 and 6 primer set: hER- α 4-6 (sense) 5' - CAG GGG TGA AGT GGG GTC TGC TG - 3' (priming site in exon 4, nucleotides 1060-1082 [14]); hER- α 4-6 (antisense) 5' - ATG CGG AAC CGA GAT GAT GTA GC - 3' (priming site in exon 6, nucleotides 1520-1542 [14]).

Human ER- α exon 5 and 8 primer set: hER- α 5-8 (sense) 5' - TCC TGA TGA TTG GTC TCG TCT GG - 3' (priming site in exon 5, nucleotides 1389-1411 [14]); hER- α 5-8 (antisense) 5' - CAG GGA TTA TCT GAA CCG TGT GG - 3' (priming site in exon 8, nucleotides 2035-2057 [14]).

Southern Blot Analysis: 10 μ l of non-radioactive PCR products were subjected to Southern blotting as previously described [11] and the resulting blots were hybridized with mER- α cDNA (kindly provided by Dr K Korach, NIEHS, North Carolina) labelled with 32 P by nick-translation as previously described [11]. The mER- α cDNA used, contained the entire coding sequences and represented nucleotides 177-2061 [13,15].

PCR products from human and murine tissue were subcloned into the cloning vector, pGEM-T Easy (Promega) following the manufacturer's instructions. Double stranded DNA from at least two independent clones from

each tissue was sequenced using a T7 Sequencing kit (Pharmacia) following the manufacturer's protocol. All RT-PCRs were carried out at least three times for each sample analyzed.

RESULTS.

Previously, we have used a long range RT-PCR approach to examine the pattern and relative frequency of expression of all exon deleted ER variant mRNAs as long as they also contain the primer sequences located in exons 1 and 8 [3]. Long range RT-PCR was carried out using mouse primer set 1-8 (see Figure 1A) with murine RNA and human primer set 1-8* (see Figure 1B) with human RNA. Although these primer sets are not exactly the analogous sequences between the two species they are similar in that they both prime within the first and last protein coding exons of the appropriate species ER cDNA (Figure 1). The results shown in Figure 2 indicate that one RT-PCR product of 1477 bp is detected in RNA isolated from murine ovarian and uterine tissues, this is the expected product for the wild type murine ER mRNA. No other RT-PCR products are detected in murine tissues, suggesting that little or no ER deletion variant mRNAs are expressed in murine tissues. In contrast, in human ovarian and uterine tissues several smaller RT-PCR products are clearly detected together with the 1381 bp RT-PCR product expected for the wild type human ER mRNA. In human tissues an abundant 1197 bp RT-PCR product is detected which corresponds to an exon 7 deleted ER mRNA, a less abundant 1045 bp RT-PCR product is detected which corresponds to an exon 4 deleted ER mRNA and a less abundant 861 bp RT-PCR is detected which corresponds to an exon 4 + 7 deleted ER mRNA. These data suggest that if murine tissues express ER deleted variant mRNA their relative abundance compared to wild type ER mRNA is very low, especially when compared to that seen in human tissues, even though levels of wild type ER mRNA within any one tissue e.g.

uterus, are similar between the two species analysed in this study. To further investigate possibly very low levels of expression of murine ER- α exon deleted transcripts, a series of primers were designed to amplify overlapping but smaller regions of the coding sequences of murine ER- α cDNA (Figure 1A). Exon 2-5 primers amplified an expected 675 bp product from cDNA prepared from total RNA isolated from several estrogen target tissues, murine mammary gland, ovary and uterus (Figure 3, top panel). Similarly, exon 4-7 primers amplified an expected 328 bp product (Figure 3, middle panel) and exon 6-8 primers amplified an expected 414 bp product (Figure 3, bottom panel). All these products are the predicted size for the wild type murine ER- α mRNA, although theoretically the wild type product could also include products of ER-like mRNAs which are deleted in regions outside those bounded by the PCR primer set. The specificity of all these RT-PCR products was determined by Southern blotting and hybridization with a radiolabelled murine ER- α cDNA probe (Figure 3). Therefore, these experiments detected little, if any, smaller sized PCR products which would be supportive of the expression of variant murine ER- α mRNA species deleted in exonic sequences. These data are consistent with those obtained using in the long range RT-PCR approach (Figure 2) but are in contrast to the detection of variant human ER- α mRNA species in human breast tumors using a similar technique [14].

The experiments were repeated using a radiolabelled PCR technique with no detection of deleted murine ER- α mRNA species (Figures 4, 7 & 8) except when using the exon 2-5 primer set. As shown in Figure 4, a low abundance PCR product of 338 bp and some other bands were detected in murine mammary gland, ovary and uterus only after a long exposure of the gel to X-ray film (2 days compared to 4-8 hours exposure for detection of human ER variants). The 338 bp product was that expected for an exon 4 deleted ER cDNA

and this was confirmed by cloning and sequencing. Other low abundance PCR products were also detected between the wild type PCR product and the exon 4 deleted PCR product, however, these bands did not correspond to the expected size of any exon deleted product and were not further characterized following our failure to successfully clone these products despite the parallel success with cloning the exon 4 deleted product. Since the exon 4 deleted ER transcript had been identified previously in rat brain [4] we analyzed RNA isolated from murine brain and several other tissues (Figure 5). A prominent 675 bp RT-PCR product was detected in most tissues which was consistent with a wild-type murine ER- α mRNA. A 338 bp PCR product consistent with an exon 4 deleted murine ER- α transcript and other uncharacterized bands were detected in most tissues only after prolonged exposure to X-ray film (2 days), suggesting extremely low relative levels. These data again appeared to be in marked contrast to data obtained using human breast cancer tissues [3].

For a more direct comparison we therefore determined the expression of exon deleted transcripts in RNA isolated from non-neoplastic human tissues using primer sets designed to amplify overlapping smaller regions of the coding sequences similar but not identical to those investigated in the murine experiments described above. Primer set 2-6 (Figure 6) resulted in detectable RT-PCR products, after a few hours of exposure to X-ray film, of 718 bp (exon 3 deleted), 696 bp (exon 5 deleted), 499 bp (exon 4 deleted) and 382 bp (exons 3+4 deleted) as well as the expected 835 bp product corresponding to the wild type human ER- α mRNA, in the human breast, ovarian and uterine tissues examined.

Primer sets designed to specifically detect an exon 5 deleted transcript relative to the wild-type ER- α transcript were used to compare side-by-side murine and human breast, ovarian and uterine tissues. Figure 7 shows that an

exon 5 deleted transcript (344 bp, bottom panel) is relatively highly expressed in human tissues compared to the wild type transcript (483 bp, bottom panel) but not detected in the equivalent murine tissues (Figure 7, top panel shows 328 bp for wild type transcript only).

Figure 8 shows that an RT-PCR product consistent with an exon 7 deleted ER- α mRNA (484 bp product, Fig 8 bottom panel) is easily detected in human tissues together with the expected wild type product (668 bp, Fig 8 bottom panel). However, only the expected wild type product (414 bp, Fig 8 top panel) was detected in murine tissues.

DISCUSSION.

The data presented above provide evidence that extensive alternative splicing of the estrogen receptor- α gene may be species specific. Our data show little, if any, evidence in murine tissues for the high level or the complex pattern of ER alternative splicing that is detected in human tissues [2].

Little is known about the mechanisms underlying species specific alternative splicing. However, several splicing signals [17,18], in particular exonic splicing elements [19] and different levels of some splicing factors [20,21] are likely to be involved. An investigation of murine ER- α and human ER- α exonic sequences for putative purine rich and A/C rich exonic splicing enhancer sequences [18,19] showed no major differences in frequency of detection. Differences in intronic splicing elements of the ER- α between mouse and human cannot be assessed at this time since no intronic sequence information is available for the murine ER- α and little intronic sequence information except for the intron/exon boundaries of the human ER- α is available [22].

Although only a handful of publications exist in which species specific alternative splicing of a gene has been described [23-30], two of these are also

members of the steroid hormone receptor gene family. This may suggest that species specific alternative splicing is a relatively common phenomenon within the steroid hormone receptor gene family. We found previously that an ER- β mRNA containing 54 nucleotides inserted between exon 5 and 6 sequences is found at high levels in multiple murine tissues but is not detected in the equivalent human tissues [26]. In addition, alternative splicing of exon 9 α /9 β of the glucocorticoid receptor is only detected in human tissues [25,31] and is not detected in any murine tissues studied [32]. Both I κ B β 1 and I κ B β 2 alternatively spliced isoforms are found in human cells but only I κ B β 1 was detected in murine cells [30]. As well in the mouse BRCA1 gene there is no homologue of the human exon 1 β suggesting that there is no murine equivalent to the human BRCA1 β transcript [33]. In contrast alternative spliced forms of the tumor suppressor p53 mRNA have only been detected in murine tissues and not human tissues [23]. Such data suggest either that mice and humans developed different mechanisms for whatever functions the alternative spliced products in the human subserve, or that species specific functions exist. Interestingly, functional differences between murine wild-type ER- α and human wild-type ER- α have now been reported [34]. For example, the murine ER- α displays differential induction of the lactoferrin gene when liganded with modified diethylstilbestrol agonists, however, the human ER- α was found to be activated by all these compounds with respect to activation of the lactoferrin promoter [34]. Furthermore, the activity of tamoxifen-like antiestrogens is quite different in the mouse compared to the human in particular with regard to agonist activity in the uterus [35]. However, a functional role(s) of ER- α variants in human estrogen signal transduction remains to be determined.

In conclusion, our data support a species specific difference in the expression of ER- α deletion variant mRNAs between murine and human

tissues. We cannot however, exclude the possibility that ER- α deletion variant mRNAs in the mouse are expressed in tissues at defined developmental stages or under specific pathophysiological conditions not investigated in this study. Irrespective of this, our data suggest that depending on the functional significance of ER- α deletion variants, extrapolation of results of some estrogen related studies in the mouse model to the human may be problematic.

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LEGENDS TO FIGURES.

Figure 1.

A. Schematic diagram of the wild-type murine ER- α cDNA showing the positions of the various primer sets used in PCR amplification.

B. Schematic diagram of the wild-type human ER- α cDNA showing the positions of the various primer sets used in PCR amplification.

Figure 2.

Long range RT-PCR assay of ER- α mRNA in murine ovarian and uterine tissues, and human ovarian and uterine tissues. Total RNA extracted from 2 samples of each tissue was reverse transcribed and PCR amplified using primers as shown in Figure 1. PCR amplified incorporating ^{32}P -dCTP. The PCR products were separated on a 6% denaturing polyacrylamide gel, dried and exposed to X-ray film overnight with a screen. The PCR product migrating at 1477 bp is the murine wild-type ER- α cDNA. The PCR products migrating at 1381 bp, 1197 bp, 1045 bp and 861 bp are the human wild-type, exon 7 deleted, exon 4 deleted and exon 4+7 deleted ER- α cDNAs, respectively. NC = negative control.

Figure 3.

Detection of wild-type ER- α mRNA in murine mammary, ovarian and uterine tissues. Total RNA extracted from 2 samples of each tissue was reverse transcribed and PCR amplified using primers as shown in Figure 1A. An aliquot of each PCR reaction was Southern blotted and hybridized with radiolabelled murine ER- α cDNA [13]. The blot was exposed to X-ray film for 4 hours with a screen. The PCR products migrating at sizes of 675 bp using primer set exon 2-5, 328 bp using primer set exon 4-7 and 414 bp using primer set exon 6-8 were

subsequently cloned and sequenced and identified as being wild-type murine ER- α cDNA.

Figure 4.

Detection of wild-type and exon 4 deleted ER- α mRNA in murine mammary, ovarian and uterine tissues. Total RNA extracted from 2 samples of each tissue was reverse transcribed and using primer set exon 2-5 (see Fig 1A) PCR amplified incorporating ^{32}P -dCTP. The PCR products were separated on a 6% denaturing polyacrylamide gel, dried and exposed to X-ray film for 2 days with a screen. The PCR products migrating at 675 bp and 338 bp were cloned and sequenced and confirmed to be wild type and exon 4 deleted murine ER- α cDNAs, respectively. NC = negative control.

Figure 5.

Detection of wild-type and exon 4 deleted ER- α mRNA in multiple murine tissues. Total RNA extracted from multiple murine tissues, as shown, was reverse transcribed and using primer set exon 2-5 (see Fig 1A) PCR amplified incorporating ^{32}P -dCTP. The PCR products were separated on a 6% denaturing polyacrylamide gel, dried and exposed to X-ray film for 2 days with a screen. The PCR products migrating at 675 bp and 338 bp are wild-type and exon 4 deleted murine ER- α cDNAs, respectively. NC = negative control.

Figure 6.

Detection of wild-type and exon deleted ER- α mRNAs in non-neoplastic human mammary, ovarian and uterine tissues. Total RNA extracted from 2 samples of each human tissue was reverse transcribed and using primer set exon 2-6 (see Fig 1B) PCR amplified incorporating ^{32}P -dCTP. The PCR products were separated on a 6% denaturing polyacrylamide gel, dried and exposed to X-ray film for 6-8 hours with a screen. The PCR products migrating at sizes of 835 bp,

718 bp, 696 bp, 499 bp and 382 bp were cloned and sequenced and confirmed to be wild-type, exon 3 deleted, exon 5 deleted, exon 4 deleted and exon 3+4 deleted human ER- α cDNAs, respectively. NC = negative control.

Figure 7.

Comparison of detection of exon 5 deleted ER- α cDNA in human and murine tissues. Primers specifically designed to detect only the wild-type and an exon 5 deleted ER- α cDNAs were used to PCR amplify incorporating ^{32}P -dCTP reverse transcribed RNA isolated from both human and murine mammary, ovarian and uterine tissues. Primer set exon 4-7 was used in murine samples (see Fig 1A) and primer set exon 4-6 was used in the human samples (Fig 1B). The PCR products were separated on a 6% denaturing polyacrylamide gel, dried and exposed to X-ray film for 4 hours with a screen. The results for the mouse are shown in the top panel and the results for the human are shown in the bottom panel. The 328 bp product in murine samples represents the wild-type ER- α cDNA. The faint band below the wild type product is unknown. It is not that expected for an exon 5 deleted product and has not been successfully cloned and sequenced. The results for human tissues are shown in the lower panel. The 483 bp and the 344 bp products are the human wild-type and exon 5 deleted (D5) ER- α cDNAs, respectively.

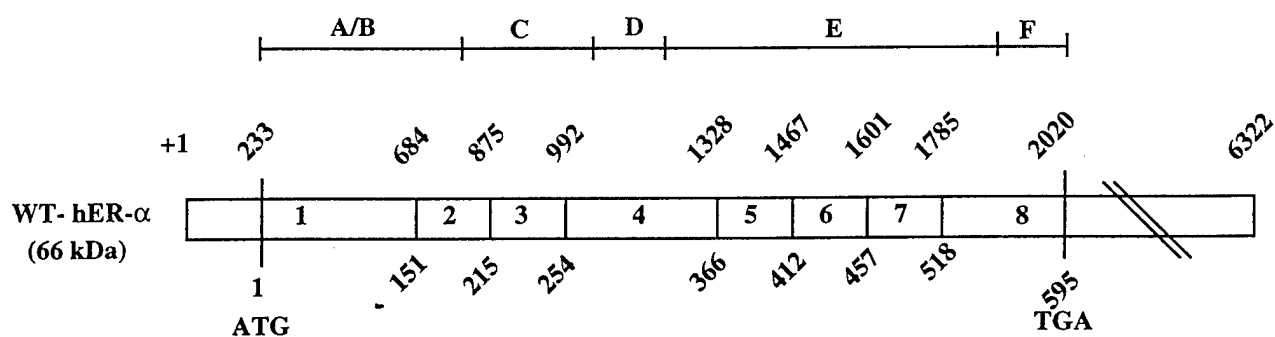
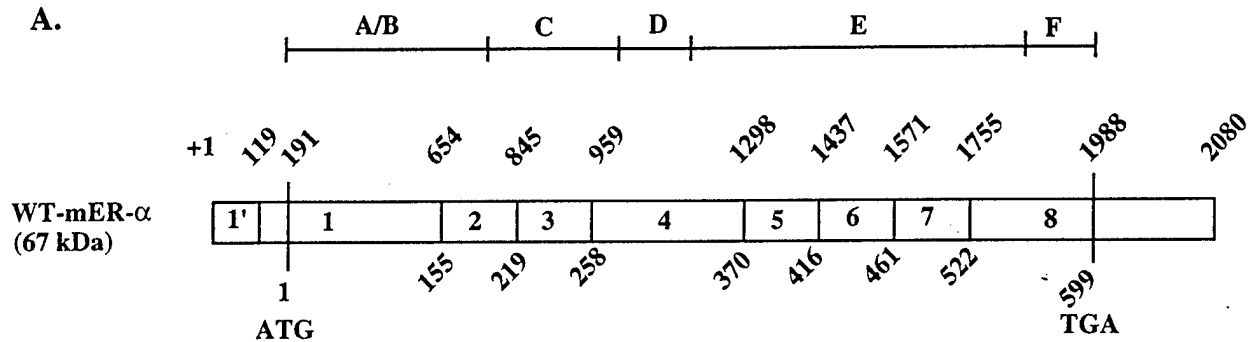
Figure 8.

Comparison of detection of exon 7 deleted ER- α cDNA in human and murine tissues. Primers specifically designed to detect only the wild-type and an exon 7 deleted ER- α cDNAs were used to PCR amplify incorporating ^{32}P -dCTP reverse transcribed RNA isolated from both human and murine mammary, ovarian and uterine tissues. Primer set exon 6-8 was used in murine samples (see Fig 1A) and primer set exon 5-8 was used in the human samples (Fig 1B). The PCR

products were separated on a 6% denaturing polyacrylamide gel, dried and exposed to X-ray film for 4 hours with a screen. The results for the mouse are shown in the top panel and the results for the human are shown in the bottom panel. The 414 bp product in murine samples represents the wild-type ER- α cDNA. The results for human tissues are shown in the lower panel. The 668 bp and the 484 bp products are the human wild-type and exon 7 deleted (D7) ER- α cDNAs , respectively.

Figure 1.

A.



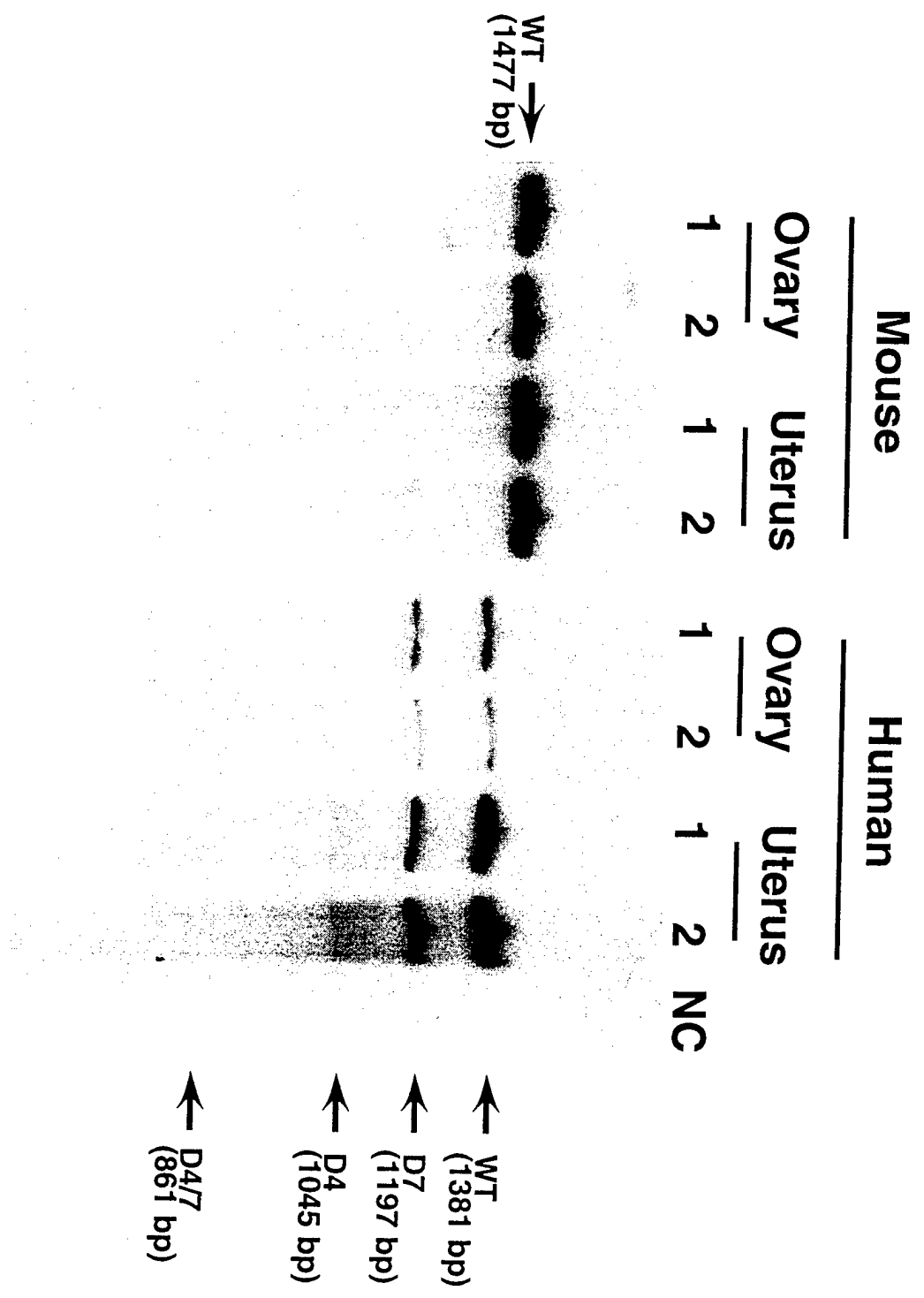
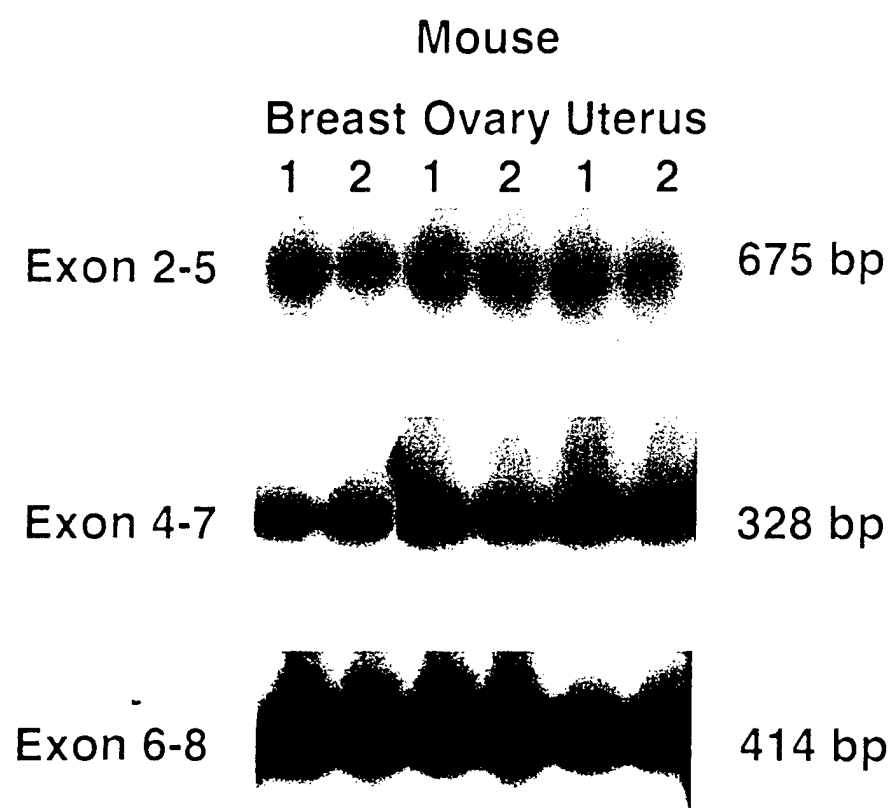
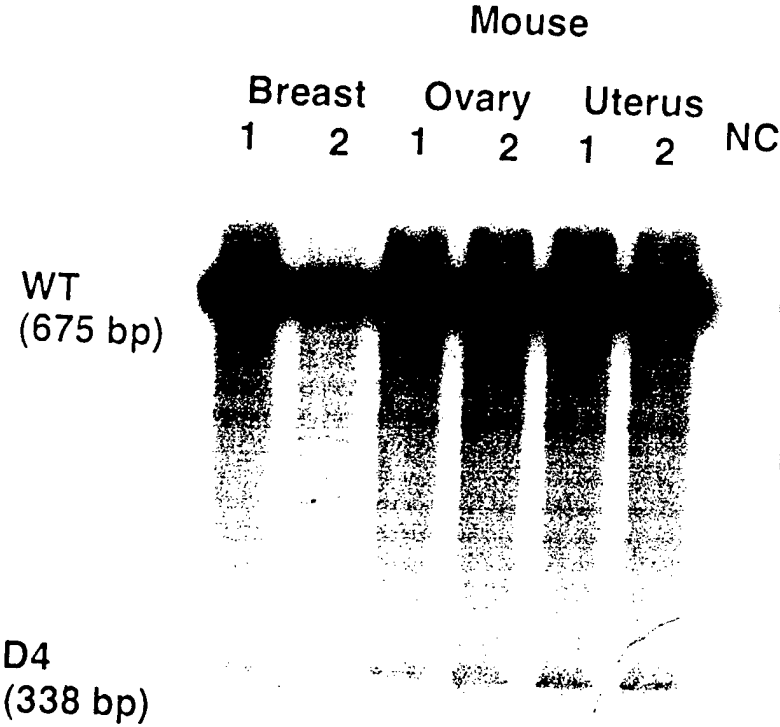


Fig 3

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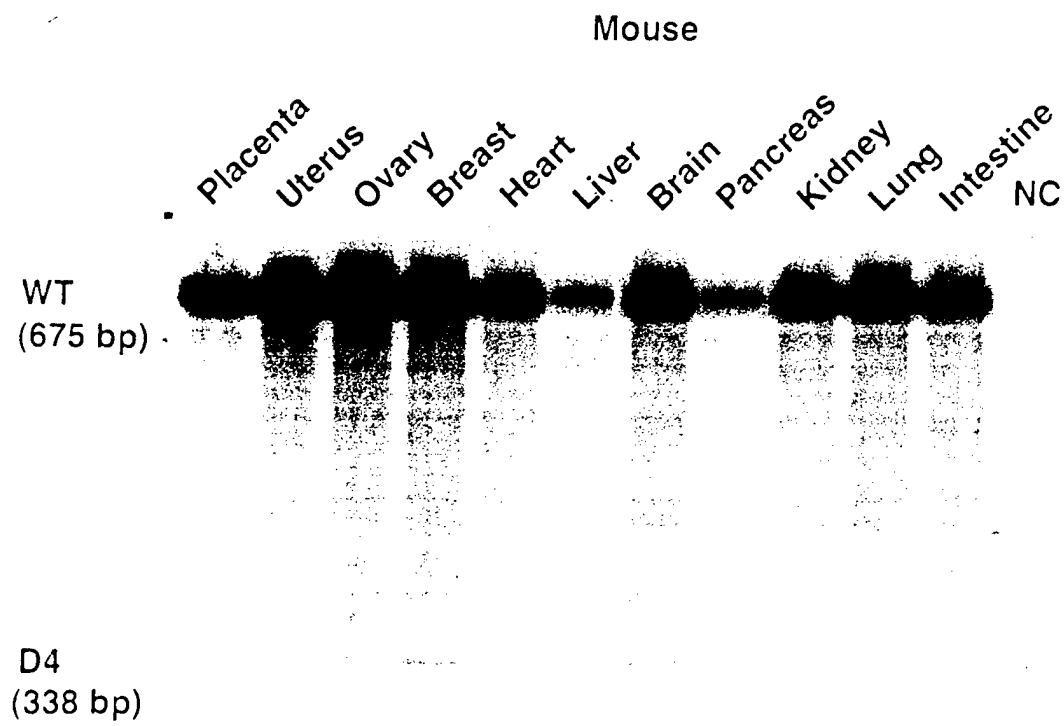
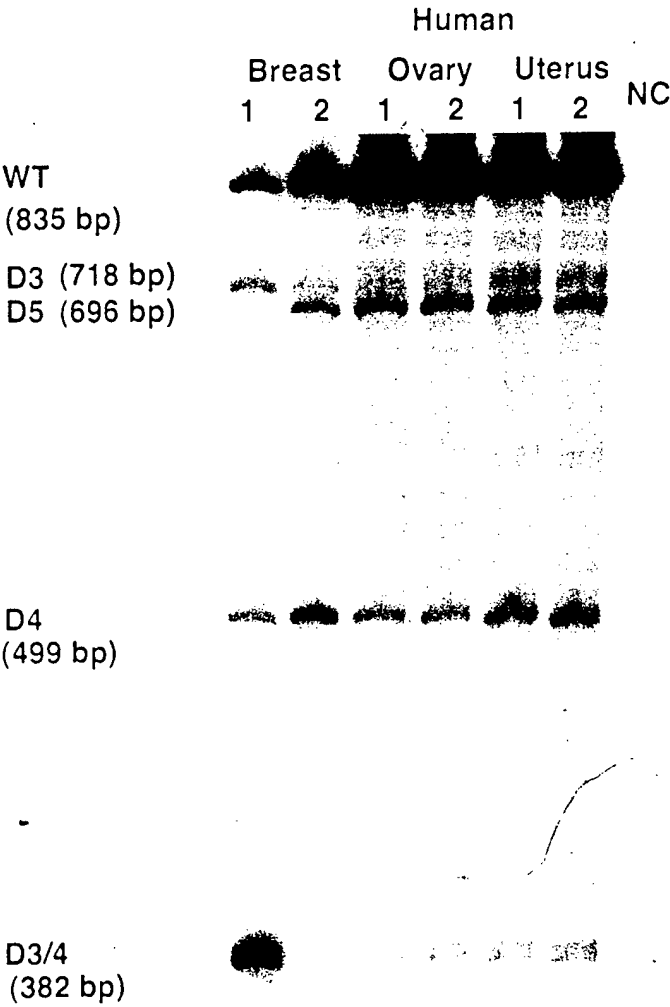


Fig 6

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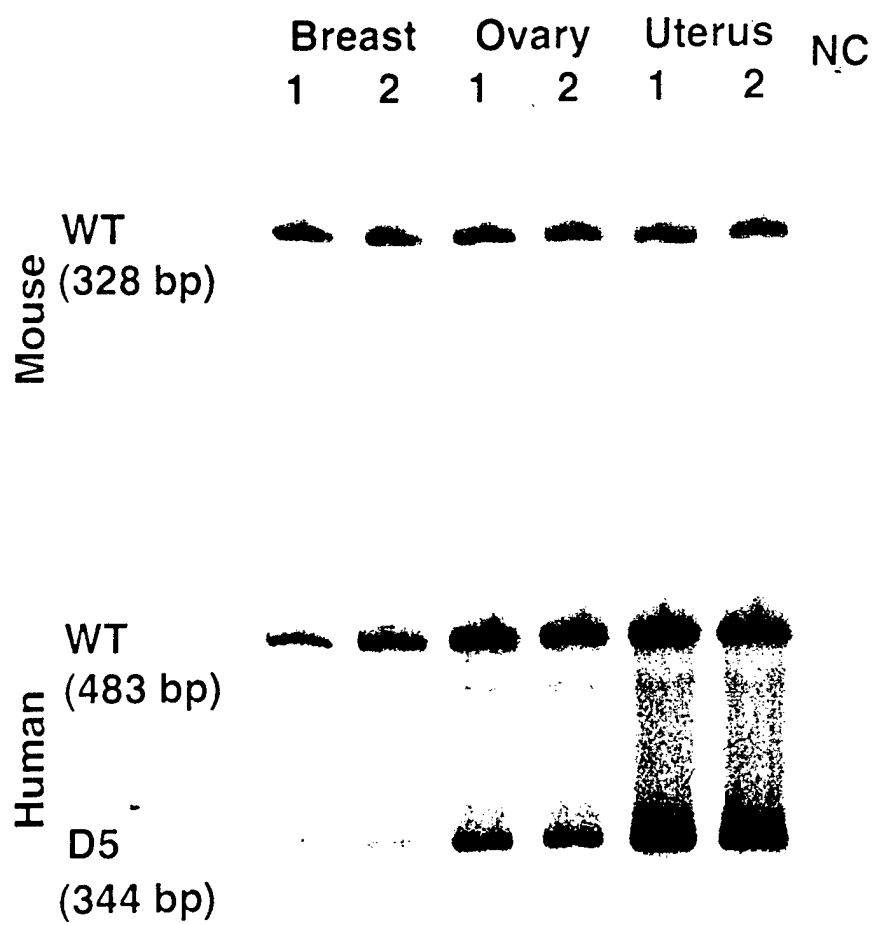
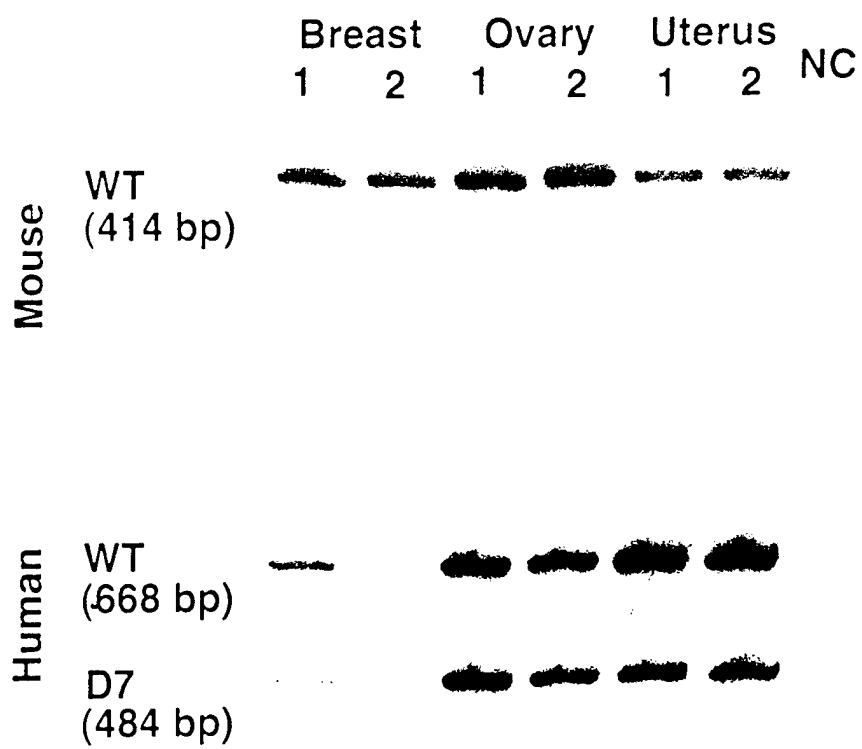


Fig 8

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APPENDIX 15

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Expression of estrogen receptor beta 1, beta 2 and beta 5 mRNAs
in human breast tissue.

Cancer Res, 59:1175-1179, 1999.

Expression of Estrogen Receptor β 1, β 2, and β 5 Messenger RNAs in Human Breast Tissue¹

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Abstract

A triple-primer PCR assay was developed, based on the coamplification of estrogen receptor (ER)- β 1, - β 2, and - β 5 cDNAs, to investigate the relative expressions of the corresponding mRNAs in breast cancer lines and in 53 independent breast tumors. The expression of ER- β 2 and ER- β 5 mRNAs was higher than that of ER- β 1 mRNA in both cancer cell lines and breast tumors. In breast tumors, increases in the ER- β 2:ER- β 1 and ER- β 5:ER- β 1 mRNA expression ratios were observed, which positively correlated with the level of tumor inflammation and tumor grade, respectively. A trend toward an increase of these ratios was also found in tumors, as compared to the normal adjacent breast tissue available for 13 cases. Our data suggest that changes in the relative expression of ER- β 1, - β 2, and - β 5 mRNAs occur during breast tumorigenesis and tumor progression.

Introduction

Estrogens regulate the growth and development of normal human mammary tissue and are also involved in breast tumor progression (1). Indeed, estrogens are thought to promote the growth of breast tumors through their mitogenic effects on breast cancer cells. The ability of antiestrogens such as tamoxifen or raloxifene to inhibit estrogenic action provides the basic rationale for the use of endocrine therapies. Estrogen action is believed to be mediated mainly through two ERs³: ER- α (2) and ER- β 1 (3, 4). These two receptors, which are encoded by two different mRNAs containing eight exons each (5, 6), belong to the steroid/thyroid/retinoic acid receptor superfamily (7). ER- α and ER- β 1 share the same structural and functional domain composition (8), defined as region A-F (Fig. 1A). The A-B regions contain the NH₂-terminal transactivation function (AF-1) of the receptors, whereas the C region of the molecule contains the DNA binding domain. The ligand binding domain and the second transactivation function (AF-2) are located within the E region of the receptors. The receptors, once bound to the ligand, are subject to conformational changes that result in complexes containing dimers of receptors/hormones that recognize estrogen-responsive elements located upstream of target genes. Interactions between ERs and accessory proteins ultimately lead to the modification of the transcription of these genes (9). The ER-ligand complexes can also interact with *c-fos/c-jun* complexes to modify the transcription of target genes through AP1 enhancer elements (10, 11). Differential activation of ER- α and ER- β 1 by the antiestrogen 4-hydroxytamoxifen, determined by acti-

vation of estrogen response element-regulated reporter genes, and differential activation of AP1-regulated reporter genes by the two ERs have been observed (11, 12). Also, because heterodimerization of ER- α and ER- β 1 has also been shown, putative cross-talk between the two signaling pathways is possible (4, 13).

Several variant forms of ER- α and ER- β 1 mRNAs have been identified (for reviews see Refs. 14-17). Among them, exon-deleted variant mRNAs, which would encode ER-like proteins missing some of the functional domains of the wild-type receptors, could interfere with ER- α and/or ER- β 1 signaling pathways. Indeed, exon 5- and exon 7-deleted ER- α variant proteins have been shown, *in vitro*, to exhibit a constitutive transcriptional (18) and a dominant negative activity (19) on ER- α , respectively. More recently, an ER- β 2 variant, deleted of regions encoded by ER- β 1 exon 8 sequences, has been shown to heterodimerize with both ER- β 1 and ER- α and to inhibit ER- α DNA binding capability (20, 21). The ability of ER- α variants to potentially interfere with the ER- α signaling pathways raised the question of their possible involvement in mechanisms underlying breast tumorigenesis and tumor progression. Although much data have been published documenting the differential expression of ER- α variants at different stages of breast cancer progression (14), no studies have been performed comparing the relative expression of ER- β variant mRNAs in human breast tissue. We have developed a TP-PCR assay to evaluate the relative expression of ER- β 1, - β 2, - β 4, and ER- β 5 variant mRNAs. As shown in Fig. 1A, ER- β 2, - β 4, and - β 5 variant mRNAs do not contain exon 8 ER- β 1 sequences but share similar 3' end sequences. This assay was used to evaluate the relative expression of ER- β 1, - β 2, and - β 5 mRNA within breast tumors ($n = 53$) and, in some cases ($n = 13$), within adjacent normal breast tissue.

Materials and Methods

Human Breast Tissues and Tumor Cell Lines. Fifty-three cases were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As reported previously, all cases in the bank have been processed to provide paraffin-embedded tissue blocks and mirror-image frozen tissue blocks (22). Histopathological analysis was performed on H&E-stained sections from the paraffin tissue block to estimate, for each case, the proportions of tumor and normal epithelial cells, fibroblasts, and fat as well as to determine the levels of inflammation and Nottingham grade scores (23). The age of the patients ranged between 39 and 87 years ($n = 53$, median = 67 years). Tumors spanned a wide range of ER (from 0 to 159 fmol/mg protein, $n = 53$, median = 9 fmol/mg protein) and PR (ranging from 0 to 285 fmol/mg protein, $n = 53$, median = 10 fmol/mg protein) levels, as measured by ligand binding assay. These tumors also covered a wide spectrum of grades (Nottingham grading scores from 1 to 9, $n = 47$, median = 7). Inflammation levels were assessed for 51 cases by scoring the extent of lympho-histocystic infiltrates throughout the section using a semiquantitative scale from 0 (low to minimal infiltration) to 5 (marked infiltrate). For 13 cases, matched adjacent normal tissue blocks were also available. The characteristics of this subset of 13 tumors were as follows: ER status ranged from 0 to 159 fmol/mg protein (median = 3.5 fmol/mg protein), PR status ranged from 4.9 to 134 fmol/mg protein (median = 8.5), Nottingham grade scores ranged from 5 to 9 (median = 7), inflammation levels ranged from 1 to 5 (median = 3), and patients were between 39 and 75 years old (median age = 54 years).

MDA-MB-231, MDA-MB-468, ZR-75, BT-20, T-47D, and MCF-7 breast

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³ The abbreviations used are: ER, estrogen receptor; TP-PCR, triple-primer PCR; PR, progesterone receptor.

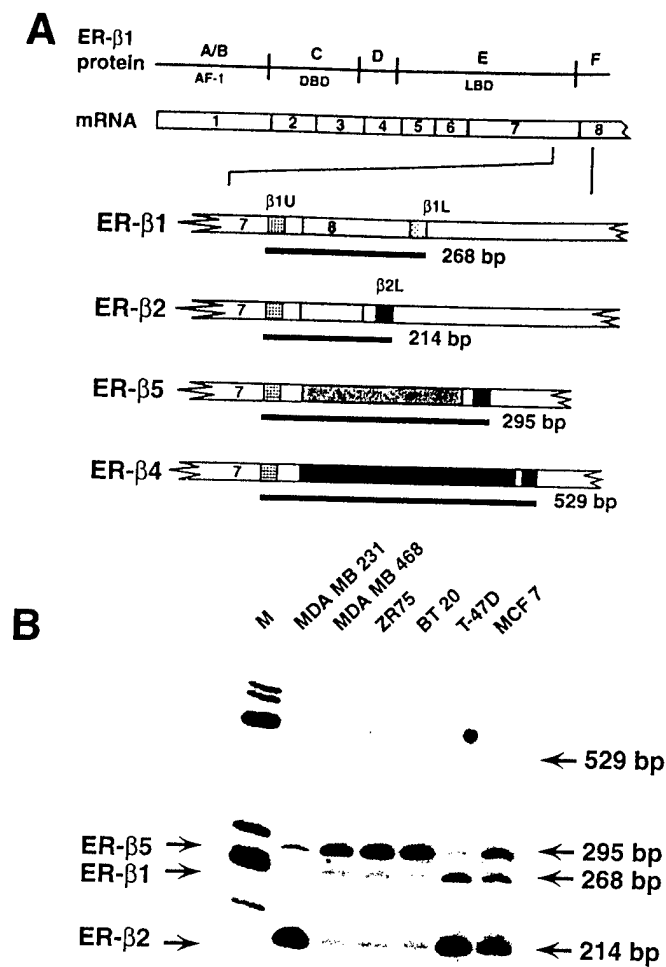


Fig. 1. Presentation of the TP-PCR assay and relative expression of ER- β 1, - β 2, - β 4, and ER- β 5 mRNAs in breast cancer cell lines. A, structural and functional domains (AF) of the ER- β 1 protein (AF-1, transactivation function 1; DBD and LBD, DNA and ligand binding domains, respectively) are shown together with the corresponding exonic structure (exons 1–8) of the ER- β 1 mRNA. Common sequences (\square) and specific sequences (\square) are depicted for each cDNA (β 1, β 2, β 4, and β 5). ER- β 1U (\blacksquare), ER- β 1L (\square), and ER- β 2L (\blacksquare) primer annealing sites are also represented. The sizes of the possible PCR products (black bars) obtained after TP-PCR are indicated. B, breast cancer cell line (MDA-MB-231, MDA-MB-468, ZR-75, BT-20, T-47D, and MCF-7) poly(A) mRNAs were reverse-transcribed, TP-PCR was performed, and PCR products were separated on an acrylamide gel, as described in "Materials and Methods." PCR products migrating at apparent sizes of 295, 268, and 214 bp have been subcloned, sequenced, and identified as corresponding to ER- β 5, - β 1, and - β 2 mRNAs, respectively. Lane M, molecular size markers (ϕ x174 RF DNA/Hae III fragments; Life Technologies, Inc.).

cancer cells were grown and poly(A) mRNA was obtained as described previously (24). Total RNA was extracted from frozen breast tissue sections using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions, and quantified spectrophotometrically. One μ g of total RNA was reverse-transcribed in a final volume of 25 μ l as described previously (25).

Primers and PCR Conditions. The primers used consisted of ER- β 1U primer (5'-CGATGCTTTGGTTGGGTGAT-3'; sense, located in exon 7, positions 1400–1420, GenBank accession no. AB006590), ER- β 1L primer (5'-GCCCTCTTTGCTTTACTGTC-3'; antisense, located in exon 8, positions 1667–1648, GenBank accession no. AB006590), and ER- β 2L (5'-CTT-TAGGCCACCGAGTTGATT-3'; antisense, located in ER- β 2 extrasequences, positions 1933–1913, GenBank accession no. AF051428). PCR amplifications were performed, and PCR products were analyzed as described previously, with minor modifications (25). Briefly, 1 μ l of reverse transcription mixture was amplified in a final volume of 15 μ l, in the presence of 1 μ Ci of [α - 32 P]dCTP (3000 Ci/mmol), 4 ng/ μ l each primer (ER- β 1U, ER- β 1L, and ER- β 2L), and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 60°C, 30 s at 72°C, and 30 s at 94°C). PCR

products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase cDNA was performed in parallel, and PCR products, separated on agarose gels, were stained with ethidium bromide as described previously (25). Identity of PCR products was confirmed by subcloning and sequencing, as reported previously (25).

TP-PCR Validation. The first series of experiments, performed using cDNAs prepared from breast cancer cell line mRNA, showed that ER- β 1, - β 2, and - β 5 cDNAs can be coamplified, and they led to the production of three PCR products that were subcloned and sequenced as described previously (25). Spiked cDNA preparations containing 1 fg of purified PCR products, corresponding to ER- β 1 and - β 5 mRNAs, were amplified together with increasing amounts of ER- β 2 PCR product (0, 0.2, 0.4, 1, 4, and 8 fg) in a single PCR tube using the three primers (ER- β 1U, ER- β 1L, and ER- β 2L), as described above. Similar experiments were performed using constant amounts of ER- β 1 and ER- β 2 or of ER- β 2 and ER- β 5, with increasing amounts of ER- β 5 or ER- β 1 PCR products, respectively. In parallel, preparations containing 1 fg of each PCR product alone were also amplified. In every case, PCR products were separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. Signals were quantified by excision of the appropriate bands and counting in a scintillation counter (Beckman). For each sample, ER- β 1, - β 2, and - β 5 signals were expressed as a percentage of the sum of all signals measured (ER- β 1 + ER- β 2 + ER- β 5 signals). Experiments have been performed in duplicate and the mean of the relative signals calculated. For each ER- β isoform, regression analyses between the relative signal obtained and the relative initial input (*i.e.*, ER- β isoform input expressed as a percentage of ER- β 1 + ER- β 2 + ER- β 5 input) were performed using GraphPad Prism software.

Quantification and Statistical Analyses. To quantitate the relative expression of ER- β 1, - β 2, and - β 5 mRNAs within each breast tissue sample, we used the TP-PCR described above. Quantification of ER- β 1, - β 2, and - β 5 signals was carried out by excision of the bands and scintillation counting. For each sample, ER- β 1, - β 2, and - β 5 signals were expressed as a percentage of the sum of all signals measured (ER- β 1 + ER- β 2 + ER- β 5 signals). Three independent PCRs were performed and the mean of the relative expressions was calculated. Differences between ER- β 1, - β 2 and - β 5 relative expression within the cohort studied were tested using the Wilcoxon signed rank test (two-tailed). Correlations with tumor characteristics were tested by calculation of the Spearman coefficient (*r*).

Results

Validation of TP-PCR as an Approach to Evaluate the Relative Expression of ER- β 1, - β 2, and - β 5 mRNAs. We established previously that TP-PCR provided a reliable method to investigate the expression of a truncated mRNA relative to the wild-type mRNA expression within small breast tissue samples (25). In its initial design, the TP-PCR assay relied on the coamplification of one truncated and a wild-type cDNA molecule using three primers in the PCR. The upper primer recognized both sequences, whereas the two lower primers recognized the variant and the wild-type sequences, respectively. We have shown that the final ratio between the two coamplified products was linearly related to the initial cDNA input (25).

As shown Fig. 1A, ER- β 1, - β 2, - β 4, and - β 5 mRNAs all have exon 7 sequences but differ from each other in the following sequences. Interestingly, comparison of the sequences revealed that ER- β 2, - β 4, and - β 5 mRNAs have sequence similarities within their 3' extremities. Therefore, it was possible to use TP-PCR to investigate the relative expression of these variants. Three primers were designed (ER- β 1U, ER- β 1L, and ER- β 2L) that recognized exon 7 sequences common to all transcripts, ER- β 1 exon 8-specific sequences, and sequences shared by ER- β 2, - β 4, and ER- β 5 mRNAs, respectively (Fig. 1A). As shown in Fig. 1A, the expected PCR products resulting from the coamplification of the corresponding cDNAs are different in size and can be easily distinguished on an acrylamide gel.

The assay was used initially to determine the expression of ER- β 1, - β 2, - β 4, and - β 5 mRNAs in several different human breast cancer cell lines (Fig. 1B). Three bands migrating at apparent sizes of 268, 214, and 295 bp were observed in all samples. Subcloning and

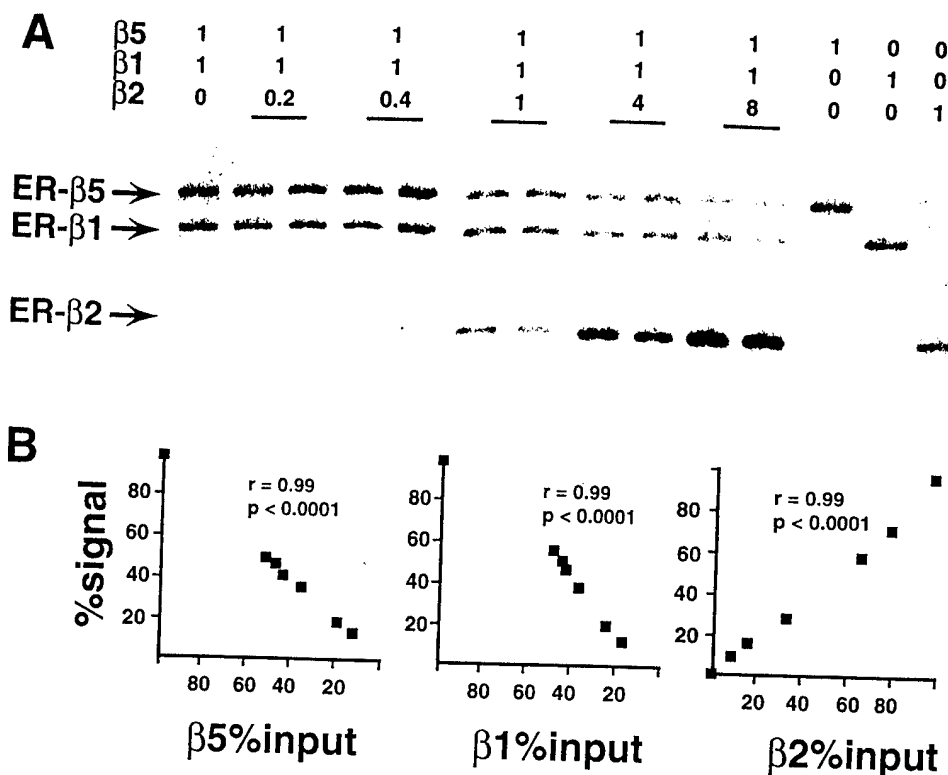


Fig. 2. TP-PCR validation. A, spiked cDNA preparations, containing various amounts [indicated above the autoradiogram] of ER- β 5, - β 1, and - β 2 purified PCR products (β 5, β 1, and β 2) were amplified by TP-PCR, and PCR products were separated on an acrylamide gel, as specified in "Materials and Methods." The autoradiogram shows the PCR products obtained. B, signals corresponding to ER- β 5, - β 1, and - β 2 PCR products have been quantified in each lane, as described in "Materials and Methods." For each ER- β isoform, the relative signal observed (percentage signal, expressed as a percentage of the sum: β 1 + β 2 + β 5 signals) is presented as a function of the initial relative cDNA input (percentage input, expressed as a percentage of the sum: β 1 + β 2 + β 5 inputs). The regression coefficient (r) and P of the associations are also presented.

sequencing of these bands confirmed their identity as ER- β 1, - β 2, and - β 5 cDNAs, respectively (data not shown). We were unable to detect a product of 529 bp, which would correspond to the ER- β 4 PCR product. Interestingly, in all tumor cell lines, the ER- β 1 signal was lower than the ER- β 2 and/or ER- β 5 signals (Fig. 1B).

Because TP-PCR performed using these primers produced three PCR products, instead of the two PCR products obtained in the original published validation studies (25), it was necessary to establish the quantitative relationship between the signals obtained and the initial target concentrations. To address this issue, spiked DNA preparations containing equal amounts of ER- β 1 and ER- β 5 PCR products and increasing amounts of ER- β 2 PCR products were amplified (Fig. 2A). The relative signals of ER- β 1, - β 2, and - β 5 have been measured and expressed as a percentage of the sum of the signals measured, as described in "Materials and Methods." As expected, in the absence of ER- β 2, only two bands, corresponding to ER- β 1 and ER- β 5 PCR products, are observed. The relative signals of ER- β 1 and ER- β 5 decreased, whereas the ER- β 2 relative signal increased linearly with increasing ER- β 2 input. Indeed, for each ER- β isoform, regression analysis showed a linear correlation between the relative signal of the PCR product measured and its relative input (Fig. 2B). Similar results were obtained when experiments were performed using constant amounts of ER- β 1 and ER- β 2 with increasing amounts of ER- β 5 PCR products or using constant amounts of ER- β 2 and ER- β 5 with increasing amounts of ER- β 1 (data not shown). It should be noted that the amplification of similar amounts of the three molecules led to the production of three bands of similar intensities (Fig. 2A). It should also be stressed that the ER- β 5:ER- β 1 ratio was not affected by increasing amounts of ER- β 2 and that the ER- β 2:ER- β 5 and ER- β 5:ER- β 2 ratios varied as a linear function of the initial ER- β 2:ER- β 5 and ER- β 5:ER- β 2 input ratios, respectively (data not shown). We concluded that the TP-PCR assay, performed under the described conditions, provided a reliable method with which to compare breast tissue samples for their relative expression of ER- β 1, - β 2, and - β 5 mRNAs.

Comparison of the Relative Expression of ER- β 1, - β 2, and - β 5 mRNAs in Breast Tumor Tissues. To determine whether alterations occur in the balance between ER- β 1, - β 2, and - β 5 mRNAs during

breast tumor progression, the relative expression of these transcripts was measured in primary breast tumor tissues from 53 different patients, using the TP-PCR assay described above. These tumors presented a wide spectrum of ER and PR statuses, as determined by ligand binding assay, and also spanned a wide range of grades and inflammation levels (for a more detailed description of the cohort characteristics, see "Materials and Methods"). Total RNA was extracted from frozen tissue sections and reverse-transcribed as described in "Materials and Methods." TP-PCR was then performed. Examples of the results obtained are shown in Fig. 3A. Three PCR products migrating at apparent sizes of 268, 214, and 295 bp were obtained. These PCR products were shown by cloning and sequencing to correspond to ER- β 1, - β 2, and ER- β 5 cDNAs, respectively. As in our preliminary study performed in human breast cancer cell lines, no band of 529 bp was detected, which would correspond to ER- β 4 PCR product. Amplification of the ubiquitously expressed *glyceraldehyde-3-phosphate dehydrogenase* cDNA, performed to check the integrity of each cDNA studied, revealed similar amounts of cDNA in all samples (data not shown). ER- β 1, - β 2, and - β 5 signals obtained in three independent TP-PCRs were quantified as described in "Materials and Methods." For each sample, the percentage of each band relative to the sum of the signals obtained has been calculated. The medians of ER- β 1, - β 2, and - β 5 relative expression within tumors, sorted according to their grade or to the level of inflammation, are presented in Fig. 3B. The ER- β 1 relative signal was found to be significantly lower than ER- β 2 (Wilcoxon sign rank test, $n = 53$, $P = 0.0002$) and ER- β 5 (Wilcoxon sign rank test, $n = 53$, $P = 0.004$) signals. A trend toward a higher expression of ER- β 2 as compared to ER- β 5 was also observed but did not reach statistical significance (Wilcoxon sign rank test, $n = 53$, $P = 0.09$).

Possible associations between ER- β 1, - β 2, or - β 5 signals and tumor characteristics were then investigated. ER- β 1 relative expression was found (Fig. 3B) to be inversely related to the grade of the tumor ($n = 47$, Spearman $r = -0.33$, $P = 0.02$) and the level of inflammation ($n = 51$, Spearman $r = -0.28$, $P = 0.04$). No other associations were found between ER- β 1 expression and ER status, PR status, or age of the patients. ER- β 2 mRNA expression increased

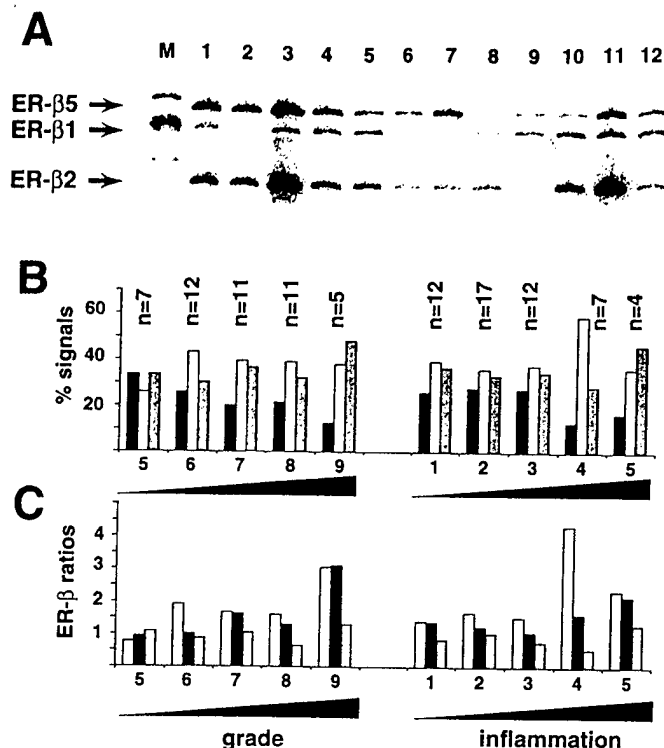


Fig. 3. TP-PCR analysis of the relative expression of ER- β 1, - β 2, and - β 5 mRNAs within a cohort of 53 independent breast tumors. Total RNA was extracted from 53 breast tumors, reverse-transcribed, and analyzed by TP-PCR, as described in "Materials and Methods." PCR products were separated on acrylamide gels. A, autoradiogram showing the results obtained for 12 cases (Lanes 1–12). Lane M, molecular weight marker ϕ x174 RF DNA/HaeIII fragments. B, ER- β 1, - β 2, and - β 5 signals have been quantified and expressed relatively to the sum of the signals obtained, as described in "Materials and Methods." Tumors have been sorted according to their Nottingham grade scores (5–9) or to their levels of inflammation (1–5). For each group, the number of patients (*n*) and the medians of the relative expression of ER- β 1 (■), - β 2 (□), and - β 5 (▨) signals are indicated. C, for each group, the number of patients (*n*) and the median of ER- β 2:ER- β 1 (□), ER- β 5:ER- β 1 (■) and ER- β 5:ER- β 2 (▨) signal ratios are indicated.

significantly with the levels of inflammation ($n = 52$, Spearman $r = 0.28$, $P = 0.04$). No other associations were found between ER- β 2 and ER- β 5 and other characteristics.

Because the ratio between two signals was related to the respective proportion of the two corresponding cDNAs, we also addressed the question of the expression of ER- β 2 and ER- β 5 relative to ER- β 1. The medians of the ER- β 2:ER- β 1, ER- β 5:ER- β 1, and ER- β 5:ER- β 2 ratios within tumors, sorted according to their grade or to the level of inflammation, are presented in Fig. 3C. ER- β 5 and ER- β 2 expression relative to ER- β 1 were found positively associated with the tumor grade ($n = 47$; Spearman $r = 0.29$, $P = 0.04$; and Spearman $r = 0.28$, $P = 0.05$, respectively). In addition, one should note that ER- β 2 expression relative to ER- β 1 expression correlated ($n = 52$, Spearman $r = 0.34$, $P = 0.01$) with levels of inflammation. ER- β 2 and ER- β 5 expression relative to each other did not correlate with grade, degree of inflammation, or any other tumor characteristics. No correlations were found between the content of the tissue sections analyzed, *i.e.*, percentage of normal cells, tumor cells, fibroblasts, or fat, and ER- β 1, - β 2, and - β 5 mRNA relative expression (data not shown).

ER- β 1, - β 2, and - β 5 mRNA Expression within Matched Normal and Tumor Compartments. To determine whether changes in the expression of ER- β 1, - β 2, and - β 5 mRNAs occur during breast tumorigenesis, we compared the relative expression of these transcripts between normal breast tissue and matched adjacent tumors. Normal adjacent breast tissue was available for 13 cases belonging to the cohort described earlier in the text. The characteristics of this tumor subset are detailed in "Materials and Methods." Total RNA was extracted, and following reverse transcription, TP-PCR was per-

formed as described in "Materials and Methods." Typical results are shown in Fig. 4A. Quantification of the signals was performed as described above. Fig. 4B shows the relative expression within tumor and adjacent normal breast tissues of ER- β 1 mRNA. A trend toward a lower ER- β 1 signal (9 of 13 cases, Wilcoxon sign rank test, $P = 0.06$) in the tumor compartment compared to the normal adjacent components was observed. In contrast, trends toward higher expression of ER- β 2 (Fig. 4C) and ER- β 5 (Fig. 4D) mRNAs relative to ER- β 1 mRNA were observed in tumor compartments (8 of 13 cases, Wilcoxon sign rank test, $P = 0.10$; and 9 of 13 cases, Wilcoxon sign rank test, $P = 0.06$, respectively).

Discussion

To evaluate the relative expression of ER- β 1, - β 2, and - β 5 mRNAs within small frozen sections of human breast tissues, we have developed an assay based on the coamplification of the corresponding cDNAs in a single tube, using three primers in the PCR. The quantitative aspect of this assay was validated using preparations containing known amounts of target cDNA. The TP-PCR approach appeared to be a reliable approach to estimate not only the relative expression of each variant within the population of ER- β molecules measured (*i.e.*, ER- β 1, - β 2, and ER- β 5 mRNAs) but also the proportion of each

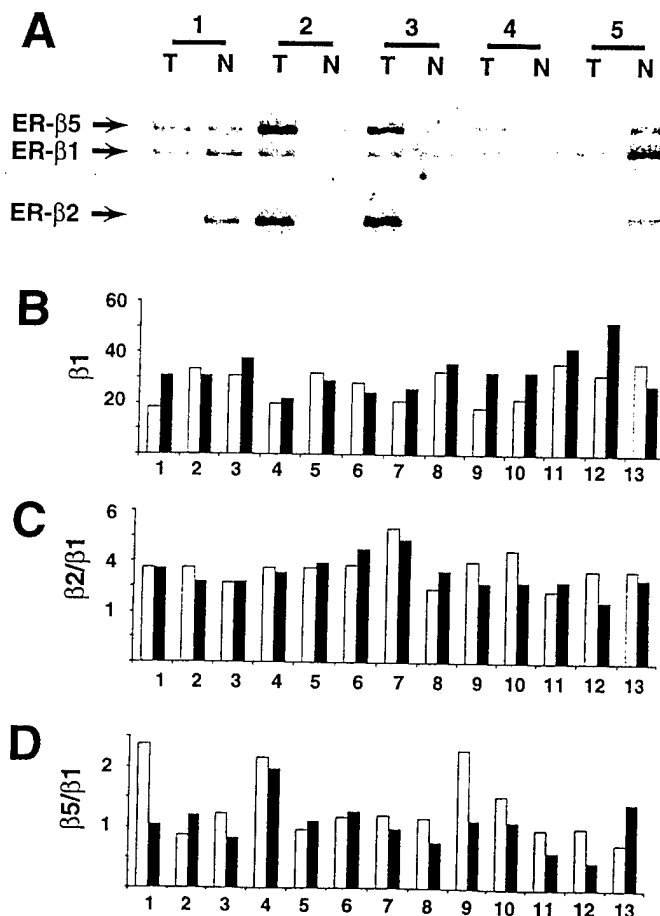


Fig. 4. TP-PCR analysis of the relative expression of ER- β 1, - β 2, and - β 5 mRNAs within matched normal and tumor compartments of human breast tumors. Total RNA was extracted from 13 breast tumors (Lanes T) and adjacent normal breast tissues (Lanes N), reverse transcribed and analyzed by TP-PCR as described in "Materials and Methods." PCR products were separated on acrylamide gels. A, autoradiogram showing the results obtained for five cases (1–5). B, ER- β 1, - β 2, and - β 5 signals have been quantified and expressed relatively to the sum of the signals obtained, as described in "Materials and Methods." For each case (1–13), the relative percentages of ER- β 1 in tumor (□) and normal (■) components are shown. C, for each case (1–13), the ER- β 2:ER- β 1 ratios in tumor (□) and normal (■) components are shown. D, for each case (1–13), the ER- β 5:ER- β 1 ratios in tumor (□) and normal (■) components are shown.

RNA relative to one another. One should note that the set of primers used would detect ER- β 4 variant mRNA. However, this variant was not detected in any breast sample or tumor cell line studied. This might result from either a lower efficiency of amplification of this specific variant in our PCR conditions or a lower relative expression of ER- β 4 mRNA as compared to ER- β 1, - β 2, and - β 5. A low expression of ER- β 4 mRNA would be consistent with data obtained in breast cancer cell lines by Moore *et al.* (20).

Our data show that ER- β 1, - β 2, and - β 5 mRNAs are coexpressed in human breast cancer cells grown in culture. These data confirm the previous observation of Moore *et al.* (20). These authors however, did not address the question of the relative expression of these mRNAs. Striking differences in the pattern of expression of ER- β 1, - β 2, and - β 5 mRNAs were found between breast cancer cell lines. If these differences in expression are conserved at the protein level, one might hypothesize that ER- β signaling pathways, which likely result from the balance between the different forms, vary in these cells. To date, multiple ER- β -like mRNAs that could encode different proteins that would be difficult to distinguish from each other by Western blot analysis have been described. For example, ER- β 1 protein (5) and ER- β 2 protein (20) have theoretical molecular masses of 54.2 kDa and 55.5 kDa, respectively. Most likely, antibodies specifically recognizing the different ER- β proteins would be the best approach to address the question of the relative expression of ER- β proteins within breast cancer cells. Higher ER- β 2 and ER- β 5 expression as compared to ER- β 1 expression was observed in breast cancer cell lines. This suggests that the respective participation of ER- β 2 and ER- β 5 variants in ER- β signaling pathways within breast cancer cells might be as significant as or more significant than that of ER- β 1.

As observed in breast cancer cell lines, ER- β 1, - β 2, and - β 5 mRNAs were detected in human breast tumors. Consistent with the observations in breast cancer cell lines, ER- β 2 and ER- β 5 mRNAs were more highly expressed than ER- β 1 mRNA in these tissues. However, even though this observation may result directly from the expression of different ER- β isoforms in breast cancer cells, it may also be a consequence of the heterogeneity of the cell populations expressing ER- β molecules and present in different proportions within the tumor sample analyzed. Indeed, because lymphocytes have previously been shown to express ER- β 1, - β 2, and - β 5 mRNAs, one could speculate that infiltrating lymphocytes within the tumor might contribute to the higher level of ER- β 2 mRNA expression in tumors with higher inflammation levels. Techniques such as *in situ* hybridization or immunocytochemistry, designed to distinguish between the different ER- β isoforms, are needed to address the question of the cellular origin of ER- β isoform expressions *in vivo*.

We observed an inverse relationship between the relative expression of ER- β 1 mRNA and tumor grade. It has been shown that the Nottingham grade provides a useful marker of the length of disease free interval and overall survival (23). We have also observed a decrease of the relative expression of ER- β 1 in tumor *versus* normal adjacent components. Taken together, these data suggest that changes in the relative expression of ER- β 1, - β 2, and - β 5 mRNAs occur during breast tumorigenesis and tumor progression. Whether these changes are a cause or a consequence of tumorigenesis remains to be elucidated.

In conclusion, we have developed a TP-PCR assay allowing the investigation of the relative expression of ER- β 1, - β 2, and - β 5 mRNA in human breast tissues. In these tissues, ER- β 1 mRNA appeared to have the lowest level of expression when compared to the two other isoforms detected. We found that the relative expression of ER- β 1 was inversely related to the grade of the tumor, suggesting that it could be used as a marker of tumor progression. Moreover, a lower relative expression of ER- β 1 was observed in tumor *versus* adjacent normal breast tissues, suggesting that changes in the expression of ER- β isoforms occur during breast tumorigenesis. The cellular origin

of the expression of ER- β 1, - β 2, and - β 5 in breast tumor tissue *in vivo* remains to be determined, as does the putative role of the different ER- β variant forms in the mechanisms underlying tumorigenesis and tumor progression.

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APPENDIX 16

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Altered estrogen receptor alpha and beta expression
during human breast tumorigenesis.

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Altered Estrogen Receptor α and β Messenger RNA Expression during Human Breast Tumorigenesis¹

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Abstract

Using a multiplex reverse transcription-PCR assay, we compared the relative expression of estrogen receptor (ER) α and ER- β mRNA between adjacent samples of normal breast tissue and matched primary breast tumors obtained from 18 different patients. Within this cohort, 7 tumors were ER negative, and 11 tumors were ER positive, as determined by the ligand binding assay. No differences in the ratio of ER- α :ER- β expression were observed in the ER-negative cohort. However, in the ER-positive cohort, a significantly ($P < 0.02$) higher ER- α :ER- β ratio was observed in the tumor compared with that of the normal tissue component. Our data revealed that the increase in the ER- α :ER- β ratio was due primarily to a significant ($P < 0.05$) increase in ER- α mRNA expression in conjunction with a lower ER- β mRNA expression in the tumor compared with that of the normal compartment in some, but not all, ER-positive cases. These results suggest that the role of ER- α - and ER- β -driven pathways and/or their interaction change during breast tumorigenesis.

Introduction

Until recently, estrogen action was thought to be mediated principally through a single ER³, ER- α , a member of the steroid/thyroid/retinoic acid receptor superfamily (1). As with other members of the family, the ER- α protein consists of several structural and functional domains (A-F). The NH₂-terminal transactivation function (AF-1) of the receptor is located within the A-B regions, whereas the DNA binding, the ligand-binding domain, and the second transactivation function (AF-2) reside in the C and E regions of the molecule, respectively (2). Upon ligand binding, conformational changes occur, and two ER- α molecules complexed with the hormone bind specifically to EREs located upstream of target genes. Interactions between ER- α and accessory proteins ultimately lead to the modification of the transcription of these genes (3). Similarly, the ER- α /estrogen complex can interact with *c-fos*/*c-jun* complexes to activate the transcription of target genes through activator protein 1 enhancer elements (4). Recently, a second ER, ER- β , was identified in the human, rat, and mouse (5-7). ER- β shares a similar structural and functional composition with ER- α and was also shown to activate the transcription of target genes through similar EREs (5, 8). However, differential activation of ER- α and ER- β by the antiestrogen 4-hydroxytamoxifen has

been shown with ERE-regulated reporter genes (9), and the two ERs also show differential activation of activator protein 1-regulated reporter genes (10), suggesting different roles for these two receptors. In addition, because heterodimerization of ER- α and ER- β has been demonstrated *ex vivo*, putative cross-talk of the two signaling pathways has been suggested (11). The tissue-specific expression of ER- α and ER- β , although not identical, shows some overlap. It has therefore been speculated that estrogen action in a given tissue may depend on the relative expression of these two receptors.

The recent demonstration of ER- β expression in both human breast tumors (12-14) and normal human breast tissue (14, 15) suggests that the well-documented role of estrogen in breast tumorigenesis (16) may also involve both receptors. To determine whether altered expression of these two receptors might occur during breast tumorigenesis, we have compared the relative expression of ER- α and ER- β mRNAs in normal human breast tissues adjacent to matched primary breast tumors.

Materials and Methods

Human Breast Tissues and Cell Line. Eighteen cases were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block was determined by the histopathological assessment of sections from adjacent mirror image paraffin-embedded tissue blocks, as described previously (17). The presence of normal ducts and lobules (median $n = 6$; range, 2-13) as well as the absence of any atypical lesion was confirmed in all normal tissue specimens. Seven tumors were ER negative (ER < 3 fmol/mg protein), with progesterone receptor values ranging from 2.2-11.2 fmol/mg protein, as measured by the ligand binding assay. Eleven tumors were ER positive (range, 3.5-159 fmol/mg protein), with progesterone receptor values ranging from 5.8-134 fmol/mg protein. These tumors spanned a wide range of grade (grade, 5-9), which was determined using the Nottingham grading system. For all normal and tumor samples, the percentage of epithelial cells, stromal component, and fat has been estimated by observation of the adjacent paraffin-embedded sections. For normal tissue, the median of the percentage of epithelial cells, stroma, and fat observed within the sections was 10 (range, 5-30%), 50 (range, 5-85%), and 40% (range, 5-90%), respectively. For tumor tissues, the median of the percentage of epithelial tumor cells, normal epithelial cells, stroma, and fat within the sections was 40 (range, 20-60%), 2.5 (range, 0-10%), 37.5 (range, 20-65%), and 20% (range, 10-50%), respectively. Three tumors (T1, T2, and T3) shown in a previous study (12) to express low ER- β /high ER- α , high ER- β /low ER- α , and high ER- β /high ER- α mRNA levels, respectively, were used to validate a multiplex RT-PCR that was designed to determine the relative expression of ER- α :ER- β mRNA. MDA-MB-231 cells were grown and harvested, and the cell pellets were stored at -70°C, as described previously (12). Total RNA was extracted from 20 μ m of frozen tissue sections (15- and 5- μ m sections for normal and tumor breast tissue, respectively) or cell pellets using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. One μ g of total RNA was reverse-transcribed in a final volume of 25 μ l as described previously (12).

Primers and PCR Conditions. The primers used consisted of ER- β -U primer [5'-GTCCATCGCCAGTTATCACATC-3' (sense), located in ER- β 130-151] and ER- β -L primer [5'-GCCTTACATCCTTCACACGA-3' (anti-

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³ The abbreviations used are: ER, estrogen receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERE, estrogen-responsive elements.

sense), located in ER- β 371–352]. The nucleotide positions given correspond to published sequences of human ER- β cDNA (5). The other pair of primers used consisted of ER- α -U primer [5'-TGTGCAATGACTATGCTTCA-3' (sense), located in ER- α 792–811] and ER- α -L primer [5'-GCTCTTCCTCCTGTTTTTA-3' (antisense), located in ER- α 940–922]. The nucleotide positions given correspond to published sequences of human ER- α cDNA (1). PCR amplifications were performed, and PCR products were analyzed as described previously, with minor modifications (12). Briefly, 1 μ l of reverse transcription mixture was amplified in a final volume of 15 μ l in the presence of 1 μ Ci of [α - 32 P]dCTP (3000 Ci/mmol), 2 ng/ μ l ER- α -U/ER- α -L and/or 4 ng/ μ l ER- β -U/ER- β -L, and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed *GAPDH* cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (12). PCR products were subcloned and sequenced as described previously (12).

Multiplex PCR Validation. Total RNA was extracted from MDA-MB-231 cells previously shown to express very low ER- α but higher ER- β mRNA levels (12) and from tumors T1, T2, and T3, the characteristics of which are described above. In the first series of experiments, six cDNA preparations were prepared that contained varying percentages of MDA-MB-231 and tumor T1 cDNA by mixing 10, 8, 6, 4, and 0 μ l of MDA-MB-231 cDNA with 0, 2, 4, 6, 8, and 10 μ l of tumor T1 cDNA (0, 20, 40, 60, 80, and 100% T1 cDNA, respectively). The same experiment was reproduced using a 10-fold dilution of these six cDNA preparations. A second series of experiments was performed in which the six cDNA preparations contained a constant amount of MDA-MB-231 cDNA (5 μ l) and 0, 1, 2, 3, 4, and 5 μ l of T1 cDNA in a final volume of 10 μ l (0, 10, 20, 30, 40, and 50% T1 cDNA, respectively). A third series of experiments contained six cDNA preparations in which the amount of T1 cDNA was held constant (5 μ l) with increasing amounts of 0, 1, 2, 3, 4, and 5 μ l of MDA-MB-231 cDNA in a final volume of 10 μ l (0, 10, 20, 30, 40, and 50% MDA-MB-231 cDNA, respectively). Finally, 1 μ l of T1, T2, and T3 cDNA was amplified independently for 22, 26, 30, and 34 cycles. In every case, PCR products were separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and autoradiographed. Signals were quantified by excision of the appropriate bands, the addition of 5 ml of scintillant (ICN Pharmaceuticals, Inc., Irvine, CA), and counting in a scintillation counter (Beckman Instruments).

Quantification and Statistical Analyses. To quantitate ER- α mRNA expression relative to ER- β mRNA expression, coamplification of ER- α and ER- β cDNAs was performed using the multiplex PCR described above. Quantification of the signals was carried out by the excision of the bands corresponding to ER- α and ER- β cDNAs, the addition of scintillant, and scintillation counting. Three independent PCRs were performed. To control for variations between experiments, a value of 100% was assigned to the highest signal measured in each set of PCR experiments, and all signals were expressed as a percentage of this signal. Indeed, the same tissue sample showed the highest signal in all experiments. For each sample, the ER- α :ER- β ratio was calculated. Differences between averages of log(ER- α :ER- β) obtained for matched normal and tumor compartments were tested using the two-tailed Wilcoxon signed rank test.

Evaluation of ER- α and ER- β mRNA expression was performed by independent amplification of both ER- α and ER- β cDNA, *i.e.* using ER- α - or ER- β -specific primers. Two independent PCRs were performed. To control for variations between experiments, all signals were expressed as a percentage of the highest signal observed. In parallel, *GAPDH* cDNA was amplified, and after analysis of the PCR products on prestained agarose gels, the signals were quantified by scanning using NIH Image 161/ppc software. Each *GAPDH* signal was also expressed as a percentage of the highest signal observed in the experiment. The average of ER- α and ER- β signals was then expressed as a percentage of the *GAPDH* signal. Differences between matched normal and tumor elements were tested using the two-tailed Wilcoxon signed rank test. Correlations were tested by calculation of the Spearman coefficient (r).

Results

Multiplex PCR as an Approach to Determine the Relative Expression of ER- α and ER- β . To determine the relative expression of ER- α and ER- β mRNA within any individual sample, we used a multiplex PCR assay. In this assay, two set of primers are added to each individual PCR, thus allowing the coamplification of both ER- α and ER- β cDNA in a single tube and therefore eliminating variation introduced due to differences in cDNA loading. To determine whether the results obtained from the multiplex PCR assay directly reflected the initial ER- α :ER- β cDNA ratio, a series of preliminary experiments was conducted. In these experiments, four different cDNA preparations were used. MDA-MB-231 cells, breast tumor T1, breast tumor T2, and breast tumor T3 had been previously shown to contain high ER- β /low ER- α , very low ER- β /high ER- α , high ER- β /low ER- α , and high ER- β /high ER- α mRNA levels, respectively (12). The first experiment consisted of the multiplex amplification of spiked cDNA preparations containing various percentages of MDA-MB-231 and T1 cDNAs (Fig. 1). As shown in Fig. 1A, the PCR signal corresponding to ER- β in MDA-MB-231 cells decreased with decreasing input of MDA-MB-231 cDNA, and the ER- α signal increased with increasing input of T1 cDNA. Quantification of the ER- α :ER- β ratio signals was plotted as a function of the percentage of T1 cDNA input (Fig. 1B). A direct relationship was found. Similar results were obtained using a 10-fold dilution of the cDNA preparations (data not shown). Using a constant amount of MDA-MB-231 cDNA plus or minus increasing amounts of T1 cDNA (containing primarily ER- α cDNA), a linear increase in the ER- α :ER- β ratio with increasing ER- α input (T1 cDNA) was found (Fig. 2, A and B). An inverse but linear relationship was obtained using a constant amount of T1 cDNA and increasing amounts of MDA-MB-231 cDNA input (data not shown). Finally, the rank of ER- α :ER- β ratios in T1, T2, and

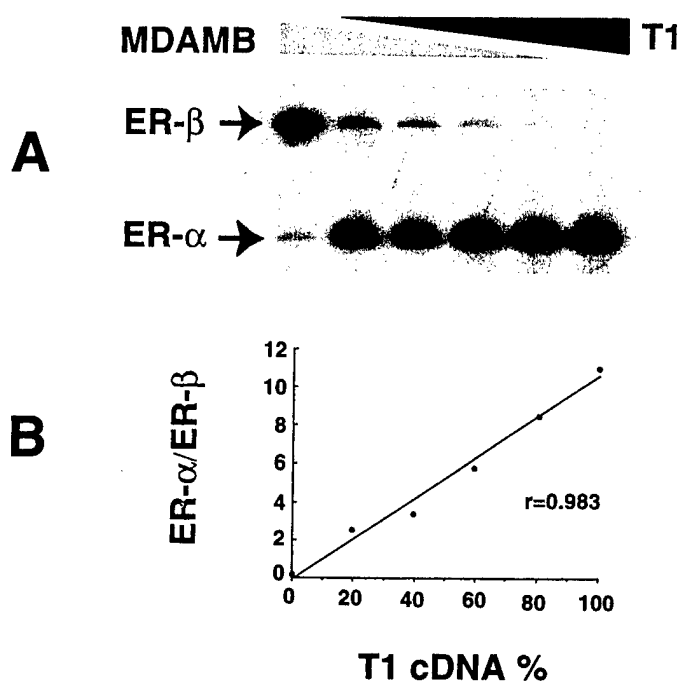


Fig. 1. Multiplex amplification of MDA-MB-231 (low ER- α /high ER- β content) and tumor T1 (low ER- β /high ER- α content) cDNA mixed preparations. An aliquot of solutions containing an increasing amount of tumor T1 cDNA and a decreasing amount of MDA-MB-231 cDNA was prepared and amplified by PCR using ER- α - and ER- β -specific primers in a single tube, and PCR products were separated on an acrylamide gel as specified in "Materials and Methods." A, autoradiograph of the gel. B, the ER- α :ER- β ratio is expressed as a function of the percentage of tumor T1 cDNA contained in the initial preparation.

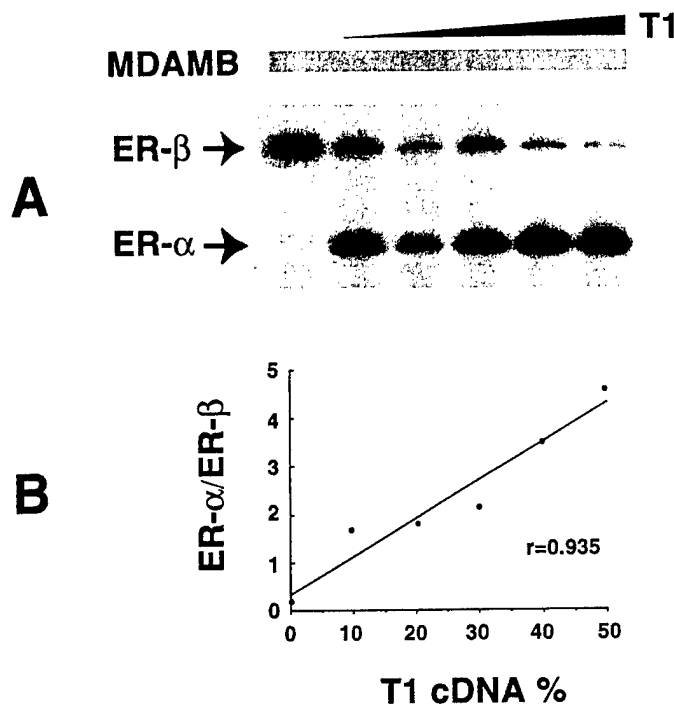


Fig. 2. Multiplex amplification of a constant amount of MDA-MB-231 (low ER- α /high ER- β content) cDNA and an increasing amount of tumor T1 (low ER- β /high ER- α content) cDNA. An aliquot of solutions containing a constant amount of MDA-MB-231 cDNA and an increasing amount of tumor T1 cDNA was prepared, amplified by multiplex PCR, and separated on an acrylamide gel as specified in "Materials and Methods." A, autoradiograph of the gel. B, the ER- α :ER- β ratio signals is expressed as a function of the percentage of tumor T1 cDNA contained in the initial preparation.

T3 using the multiplex PCR assay was not affected by the number of cycles used for the PCR over a range of 22–34 cycles. The ranking was similar to that deduced using several independent PCR determinations of the ER- α and ER- β mRNA levels, *i.e.* T1 ER- α :ER- β > T3 ER- α :ER- β > T2 ER- α :ER- β (data not shown). Multiplex PCR performed under the described conditions therefore seemed to be a reliable method with which to compare small tissue samples for their relative expression of ER- α and ER- β mRNA.

Comparison of the Relative Expression of ER- α :ER- β mRNA in Adjacent Normal Breast Tissue and Matched Primary Breast Tumors. To determine whether alterations may occur in the contribution of ER- α and ER- β signaling during breast tumorigenesis, the relative expression of ER- α and ER- β mRNA was measured in matched normal and primary tumor tissues from 18 different patients. Within the cohort of tumors studied, 7 tumors were ER negative, and 11 tumors were ER positive, as determined by the ligand binding assay. Total RNA was extracted from the frozen tissue sections and analyzed by multiplex RT-PCR. Examples of the results obtained are shown in Fig. 3A. In both normal and tumor compartments, two PCR products migrating at an apparent size of 242 and 149 bp were obtained. These PCR products were identified by cloning and sequencing to correspond to ER- β and ER- α cDNA, respectively. ER- α and ER- β signals obtained in three independent multiplex PCRs were quantified as described in "Materials and Methods." The ER- α :ER- β ratio was calculated for each sample, and the results for each matched sample are presented in Fig. 4A. Considering all cases together, no significant change in the ER- α :ER- β ratio was observed between normal and tumor compartments. The cases were then divided into two groups based on the ER positivity of their tumors. Once again,

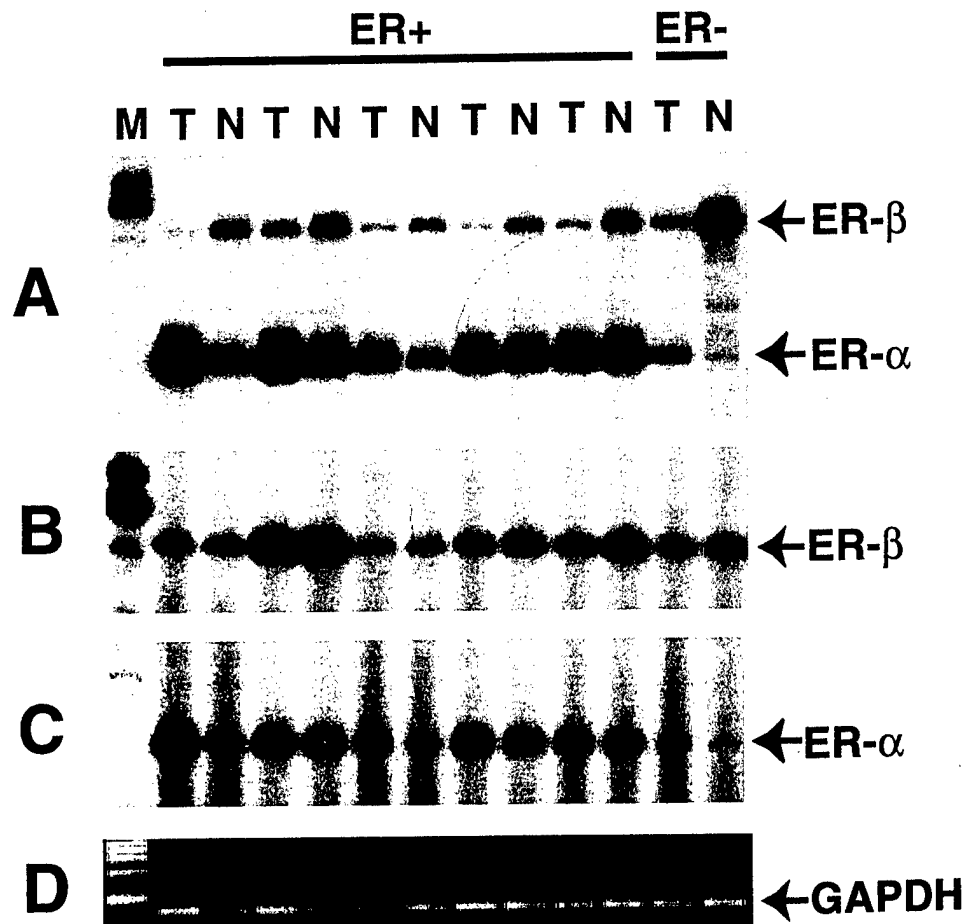


Fig. 3. RT-PCR analysis of ER- α and ER- β expression within matched normal and tumor compartments of human breast tumors. Total RNA was extracted from matched normal (N) and tumor (T) compartments of ER-positive (ER+) or ER-negative (ER-) tumors, as determined by ligand binding assay. Corresponding cDNA was amplified, and PCR products were separated on an acrylamide gel, as described in "Materials and Methods." PCR was performed using: A, ER- α and ER- β primer sets in a single tube (multiplex PCR); B, ER- β -specific primers only; C, ER- α -specific primers only. D, for each sample, GAPDH cDNA was amplified in parallel, and PCR products were separated on an agarose gel, as described in "Materials and Methods." M, molecular weight marker (ϕ x174 RF DNA/HaeIII fragments; Life Technologies, Inc.).

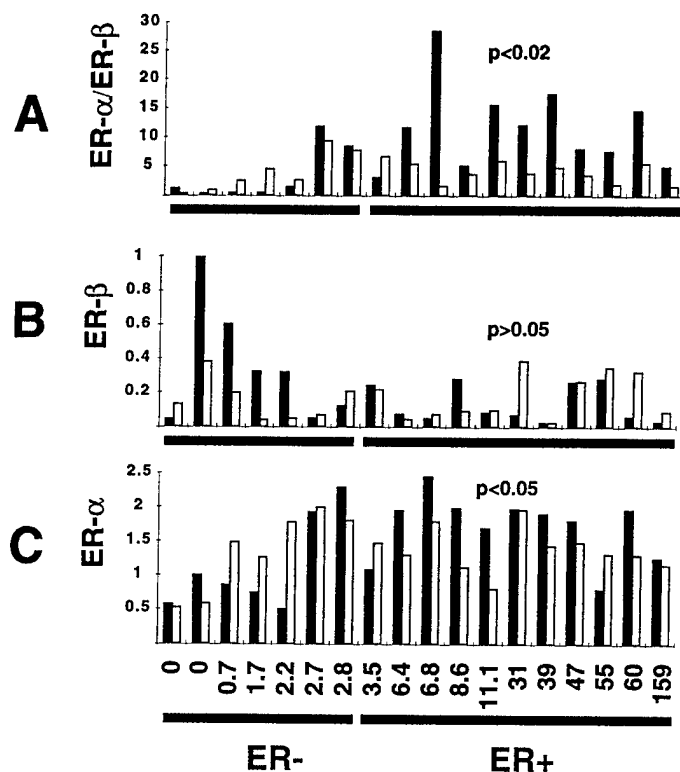


Fig. 4. Quantification of ER- α and ER- β expression within matched normal and tumor compartments of human breast tumors. Total RNA extracted from matched normal (□) and tumor (■) compartments of ER-positive (ER+) or ER-negative (ER-) tumors was reverse-transcribed and PCR-amplified, and PCR products were separated on an acrylamide gel, as described in "Materials and Methods." Signals have been quantified and normalized, as indicated in "Materials and Methods." A, ER- α :ER- β ratio obtained after multiplex PCR. B, ER- β signals obtained after PCR was performed using ER- β -specific primers only. C, ER- α signals obtained after PCR was performed using ER- α -specific primers only. ER status of the tumor component, as assessed by ligand binding assay, is indicated (fmol/mg protein). Differences between matched normal and tumor compartments in ER-positive cases were tested using the two-tailed Wilcoxon signed rank test.

within the ER-negative cohort, no difference in the ER- α :ER- β ratio was seen between normal tissue and matched tumors. In contrast, in the ER-positive tumor group, a significant increase (two-tailed Wilcoxon signed rank test, $P < 0.02$) in the ER- α :ER- β ratio was observed in the tumor compartment compared with that of the normal compartment. It should be stressed that a significant correlation was found between the ER- α :ER- β ratio observed in the tumor compartment and ER status by binding (Spearman $r = 0.603$; $P < 0.01$).

Independent Measurement of ER- α and ER- β mRNA Expression within Matched Normal and Tumor Compartments. The observed increase in the ER- α :ER- β mRNA ratio of ER-positive breast tumors *versus* matched normal tissue could result from a decrease in the absolute levels of ER- β mRNA and/or an increase in the absolute ER- α mRNA levels within the tumor compartment relative to the matched normal tissue. To distinguish between these possibilities, the ER- β and ER- α mRNA levels were determined individually in each sample by RT-PCR, using either ER- β -specific primers or ER- α -specific primers. Examples of the results obtained are shown in Fig. 3, B and C, respectively. In parallel, amplification of the ubiquitously expressed *GAPDH* cDNA was also performed (Fig. 3D). For each sample, the ER- β and ER- α cDNA signals were quantified, and the average of signals obtained in two independent PCRs was normalized to the *GAPDH* signal, as described in "Materials and Methods." The results are shown in Fig. 4, B and C, for ER- β and ER- α , respectively. No significant change in ER- β or ER- α mRNA expression was observed between the normal and tumor compartments within the ER-negative cases. Although the difference

did not reach statistical significance ($P > 0.05$), ER- β mRNA expression was higher in the normal compartment *versus* the matched tumor component in 8 of 11 (72%) ER-positive cases. A significant (two-tailed Wilcoxon-signed rank test, $P < 0.05$) increase in ER- α mRNA expression was measured in the tumor compartment of ER-positive tumors compared with that of the matched normal tissues. ER- α and ER- β signals observed in normal or tumor compartments did not correlate with the cellular composition of the section analyzed, *i.e.* percentage of normal epithelial cells, tumor epithelial cells, stroma, or fat (data not shown). One should note that although not statistically significant, trends toward an association between ER status by binding and ER- α (Spearman $r = 0.397$; $P = 0.10$) and ER- β (Spearman $r = -0.4254$; $P = 0.07$) have been observed.

Discussion

The discovery of the expression of a second ER in both normal and neoplastic human mammary tissues (12–15), together with the known perturbations of estrogen and antiestrogen sensitivity during breast tumorigenesis and breast cancer progression (16, 18–21), necessitates an investigation of the function of ER- β in human mammary tissue and a reevaluation of the estrogen signal transduction system in these tissues. We have used a multiplex assay in which ER- α and ER- β cDNA are amplified in the same reaction to investigate the relative expression of ER- α and ER- β mRNAs between adjacent samples of normal breast tissue and matched primary breast tumors. The choice of an RT-PCR-based approach to address the question of the relative expression of both receptors has been dictated by several parameters: (a) the absence of any publication to date using antibodies to detect ER- β protein in human breast tissue suggests that reliable antibodies are not yet available for this purpose; and (b) the expression of ER- β mRNA is relatively low in breast tissue, as demonstrated by the time needed to observe a signal in epithelial human breast cells in *in situ* hybridization studies (14) and by much weaker signals obtained, compared with ER- α , when analyzing breast tissue samples by RNase protection assay.⁴ The multiplex PCR assay developed here seems to be a reliable method with which to compare tissue samples for their relative expression of ER- α and ER- β mRNA. It should be stressed, however, that despite the good overall correlation coefficient observed, samples with an ER- α :ER- β ratio of <2 may be less reliably compared with each other (Fig. 2). This could possibly be a limitation of the multiplex approach, which would likely have a higher impact when comparing ER-negative tumors, in which ER- α is known to be weakly expressed. Such a limitation of multiplex PCR analysis of genes expressed at very low levels has previously been reported and may be circumvented by increasing cDNA input (22).

Our data show that in the cohort of patients whose tumors are ER positive by ligand binding, the ratio of ER- α :ER- β is significantly higher in breast tumors than it is in adjacent normal tissues. This difference seems mainly due to an up-regulation of ER- α mRNA levels within the tumor compartment. This observation is in agreement with previous published data showing a generally higher expression of ER- α detected immunohistochemically in ER-positive breast tumors than in normal breast tissue (see Ref. 23 and references herein). However, it is possible that down-regulation of ER- β expression in the tumor tissue may also contribute to the altered ratio in some tumors. Although the difference did not reach statistical significance, 72% of the ER-positive cohort showed a trend in which ER- β expression was lower in the tumor when compared with the normal compartment. The study of larger numbers of cases will be necessary to confirm this trend. Similarly, although no correlations have been

⁴ Unpublished observation.

observed between the expression of ER- α and ER- β assessed by targeted PCR and the cellular composition of the sections analyzed, one cannot exclude the possibility that such relationships might exist. The study of a larger number of samples will also clarify this issue. If these RNA studies are paralleled at the protein level, then our data suggest that a significant change in the ratio of these two ERs occurs between normal and neoplastic breast tissues. This would further suggest that the contribution of ER- α - and ER- β -driven pathways and/or their interactions changes in conjunction with breast tumorigenesis. The hypothesis that such changes in ER- α and ER- β signaling pathways may occur during tumorigenesis is also supported by the recent observations of Brandenberger *et al.* (24). These authors showed that ER- α mRNA expression is equal or slightly higher in ovarian cancer tissues compared with normal ovary tissues, and ER- β mRNA expression is decreased in ovarian tumor tissue. The measurement of the ER- α :ER- β ratio correlated with ER status as assessed by ligand binding assay. Moreover, trends toward a positive correlation between ER- α and ER status and toward a negative correlation between ER- β and ER status were observed. Together, these data suggest that ligand binding is mainly due to the ER- α protein.

We have previously observed that the apparent ER- α :ER- β ratio in breast tumors varies widely (12). Our current results using the multiplex RT-PCR approach confirm and support these previous observations. Given the differential activity of tamoxifen-like antiestrogens through ER- α and ER- β , it is tempting to speculate that altered ratios of these receptors may be a possible mechanism associated with tamoxifen resistance.

In conclusion, our results provide evidence to support the hypothesis that altered ER- α and ER- β expression may have a significant role in alterations of estrogen action that occur during human breast cancer.

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APPENDIX 17

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Expression of the steroid receptor RNA activator (SRA)
in human breast tumors.

Cancer Res, In press.

EXPRESSION OF THE STEROID RECEPTOR RNA ACTIVATOR (SRA) IN HUMAN BREAST TUMORS¹

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Key words: SRA, steroid receptor co-activator, human breast tumor, tumor progression, PCR.

Footnotes:

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³ The abbreviations used are: SRA, steroid receptor RNA activator; ER, estrogen receptor; PR, progesterone receptor; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

⁴ (unpublished data, see <http://www.ncbi.nlm.nih.gov/genemap/loc.cgi?ID=12637>).

Abstract

The expression of the recently described steroid receptor RNA activator (*SRA*) was measured by semi-quantitative reverse-transcription polymerase chain reaction within 27 independent breast tumors, spanning a wide spectrum of grade, estrogen receptor (ER) and progesterone receptor (PR) levels. Subgroup analysis showed that *SRA* expression was similar in ER+/PR+ (median = 65.5, n = 8) and in ER-/PR- (median = 94.6, n = 5) tumors. Interestingly, *SRA* expression in these two subgroups was significantly (Mann-Whitney rank-sum test, $p < 0.05$) lower than that observed in ER+/PR- (median = 156.4, n = 6) and ER-/PR+ (median = 144.8, n = 8) tumors. A variant form of *SRA*, presenting a deletion of 203 bp within the *SRA* core sequence, was also observed in breast tumor tissues. The relative expression of this new *SRA* isoform correlated with tumor grade (Spearman coefficient $r = 0.53$, $n = 27$, $p = 0.004$). These data suggest that changes in the expression of *SRA* related molecules occur during breast tumor progression.

Introduction

Estrogens, through their mitogenic action on breast epithelial cells, regulate the growth and the development of normal as well as neoplastic human mammary tissue (1). The ability of antiestrogens such Tamoxifen or Raloxifene to antagonize this estrogenic action provides the basic rationale for endocrine therapy and prevention (for a review see 2). Estrogen action is mainly mediated through two estrogen receptors, estrogen receptor alpha and beta (3-5), that belong to the steroid/thyroid/retinoic acid receptors superfamily (6) and act as ligand-dependent transcription factors. The mechanisms by which steroid receptors modulate the transcription of target genes is under extensive investigation (7). Once bound to the ligand, the receptors undergo conformational changes and dimers of receptors recognize specific regulatory DNA sequences upstream of target genes. Activated receptors, through interactions with co-activator proteins, direct the assembly and the stabilization of a preinitiation complex that will ultimately conduct the transcription of these genes (see 8 and references herein). To an already long list of nuclear receptor co-activators (8), that includes the p160 proteins (such as *SRC-1* and *AIB1*), Lanz et al. (9) recently added the steroid receptor RNA activator (*SRA*).³ *SRA* differs from other co-activators in two main features. Firstly, *SRA* transcripts do not appear to be translated and therefore, this co-activator acts as an RNA and not as a protein. Lanz et al. showed that *SRA* exists in a ribonucleoprotein

complex that contains SRC-1 and is recruited by steroid receptors (9). Secondly, *SRA* appears to be actually specific for steroid receptors. Indeed, most of the receptor-interacting factors, such as *SRC-1* or *TIF2/hSRC-2*, interact with and co-activate both class I and class II nuclear receptors (9). Because of the importance of estrogen receptor signaling pathways in the mechanisms underlying breast tumor progression, it was important to establish whether *SRA* expression could be expressed in breast tumors. If so, it was also of interest to determine if the expression of *SRA* was related to known markers of endocrine sensitivity, and prognostic markers. We have selected a subset of breast cancer cases to look for possible correlations between *SRA* expression and already established predictive and/or prognostic factors such as grade, ER and progesterone receptor (PR) status (10).

Materials and methods

Human breast tumors.

Twenty seven cases were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected according to their ER and PR status, as determined by ligand binding assay. Tumors were classified as ER -/ PR + (n = 8; ER range, 5-9 fmol/mg protein; PR range, 51-271 fmol/mg protein), ER +/PR - (n = 6; ER range, 59-151 fmol/mg protein; PR range, 5-10 fmol/mg protein), ER -/ PR - (n = 5; ER range, 0-2 fmol/mg protein; PR range, 0-8 fmol/mg protein) and ER +/PR + (n = 8; ER range, 50-127 fmol/mg protein; PR range, 101-285 fmol/mg protein). These tumors covered a wide spectrum of grade (grade from 4 to 9), determined using the Nottingham grading system (11). Patients were 49-87 years old.

RNA extraction and RT-PCR.

Total RNA was extracted from frozen breast tissue sections using Trizol™ reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions, and quantified spectrophotometrically. One µg of total RNA was reverse-transcribed in a final volume of 25 µl as previously described (12).

Primers and PCR conditions.

The primers used consisted of *SRA*coreU primer (5'-AGGAACGCGGCTGGAACGA-3'; sense; position 35-53, Genbank accession number AF092038), and *SRA* core lower primer (5'-AGTCTGGGGAACCGAGGAT-

3'; antisense; position 696-678, Genbank accession number AF092038). PCR amplifications were performed and PCR products analyzed as previously described with minor modifications (12). Briefly, 1 μ l of reverse transcription mixture was amplified in a final volume of 15 μ l, in the presence of 1.5 μ Ci of [α - 32 P] dCTP (3000 Ci/mmol), 4 ng/ μ l of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY). Each PCR consisted of 30 cycles (30 sec at 60°C, 30 sec at 72°C and 30 sec at 94 °C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and exposed 1 hour to a Molecular ImagerTM-FX Imaging screen (Bio-Rad, Hercules, CA). Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as previously described (12). Identity of PCR products was confirmed by subcloning and sequencing, as previously reported (13).

Quantification of *SRA* expression.

Exposed screens were scanned using a Molecular ImagerTM-FX (Bio-Rad, Hercules, CA) and the intensity of the *SRA* corresponding signal measured using Quantity OneTM software (Bio-Rad, Hercules, CA). Three independent PCRs were performed. In order to control for variations between experiments, a value of 100% was arbitrarily assigned to the *SRA* signal of one particular tumor (tumor 14) measured in each set of PCR experiments and all signals were expressed as a percentage of this signal. In parallel, *GAPDH* cDNA was amplified and following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using NIH Image 161/ppc software. Three independent PCRs were performed. Each *GAPDH* signal was also expressed as a percentage of the signal observed in the tumor 14. For each sample, the average of *SRA* signal was then expressed as a percentage of the *GAPDH* signal (arbitrary units).

Quantification of *SRA*-Del relative expression.

It has previously been shown that the co-amplification of a wild-type and a deleted variant cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (13, 14). For each sample, *SRA*-Del corresponding signal was

measured using Quantity One™ software (Bio-Rad, Hercules, CA) and expressed as a percentage of the corresponding *SRA* signal. For each case, 3 independent assays were performed and the mean determined.

Statistical analysis

Differences between tumor subgroups were tested using the Mann-Whitney rank sum test, two-sided. Correlation between *SRA* expression and tumor characteristics was tested by calculation of the Spearman coefficient r .

Results

Detection of *SRA* and a variant mRNA deleted form (*SRA-Del*) in human breast tumor tissues.

The existence of three different *SRA* mRNAs have been reported (9). The sequences of these isoforms differ in their 5'- and 3'- terminal regions but are identical within their central region, called the core (Fig. 1). In order to investigate the expression of all described *SRA* isoforms in human breast tumor tissues, we designed primers to amplify a 662 bp long fragment encompassing almost all the *SRA* core region. Total RNA was extracted from 27 human breast tumors, reverse-transcribed, and PCR amplification was performed as described in the "Materials and Methods" section, using *SRA* core primers. A 662 bp long fragment was obtained in all samples. However, the intensity levels varied from one sample to another (Fig. 2A). This fragment was sequenced and corresponded to the *SRA* core region. The differences in *SRA* expression were unlikely to result from different cDNA input, as shown by the similar intensities of *GAPDH* signal obtained after amplifying *GAPDH* mRNA in parallel using the same cDNAs (Fig. 2B). An additional fragment, migrating at an apparent size of 459 bp was also observed in most samples. Sequencing analysis revealed that this band corresponded to a variant form of *SRA* (referred to as *SRA-Del*) deleted in 203 bp between positions 155 and 357 (positions given correspond to Genbank accession number AF092038).

The expression of *SRA* correlates with ER and PR levels in subgroups of human breast tumors.

For each case, *SRA* corresponding signal was quantified and expressed in arbitrary units, as described in the "Materials and Methods" section. Results obtained from the 27 cases, grouped according to their ER and PR levels, as determined by ligand binding analysis, are presented Fig. 3A. When the cohort of cases was

considered as a whole ($n = 27$), no correlation was observed between *SRA* expression and ER or PR levels. Indeed, similar levels of *SRA* were found in ER+/PR+ (median = 65.5, $n = 8$) and ER-/PR- (median = 94.6, $n = 5$) tumors (Fig. 3A). However, when only ER- tumors were considered ($n = 13$), a trend toward a positive correlation between *SRA* expression and PR levels was observed (Spearman coefficient $r = 0.527$, $p = 0.064$). A statistically significant (Mann-Whitney rank sum test, two sided, $p = 0.045$) higher *SRA* expression was observed in ER-/PR+ ($n = 8$, median = 144.8) than in ER-/PR- tumors (Fig. 3A). In contrast, within ER+ cases ($n = 14$), *SRA* expression negatively correlated with PR levels (Spearman coefficient $r = -0.810$, $p = 0.0004$). *SRA* expression was higher (Mann-Whitney rank sum test, two sided, $p = 0.001$) in ER+/PR- ($n = 6$, median = 156.4) than in ER+/PR+ cases. In a similar way, *SRA* expression correlated positively (Spearman coefficient $r = 0.735$, $p = 0.009$) and negatively (Spearman coefficient $r = -0.532$, $p = 0.033$) with ER levels in PR- ($n = 11$) and PR + ($n = 16$), respectively. *SRA* levels were higher in ER+/PR- than in ER-/PR- tumors (Mann-Whitney rank sum test, two sided, $p = 0.017$) and in ER-/PR+ than in ER+/PR+ cases (Mann-Whitney rank sum test, two sided, $p = 0.047$). *SRA* levels of expression did not correlate with tumor grade scores (Fig. 3B).

The expression of *SRA-Del* correlates with breast tumor grade scores.

For each case, *SRA-Del* signal was measured and expressed relatively to the corresponding *SRA* signal, as described in the "Materials and Methods" section. *SRA-Del* relative signal did not correlate with ER or PR levels when the cohort of cases was considered as a whole or when analyzing ER-, ER+ and PR- subgroups. Interestingly, *SRA-Del* expression positively correlated (Spearman coefficient $r = 0.512$, $p = 0.042$) with PR levels in PR+ subgroup ($n = 16$). However, no statistically significant differences (Fig. 4A) were observed between ER-/PR+ ($n = 8$, median = 2.346), ER+/PR- ($n = 6$, median = 2.561), ER-/PR- ($n = 5$, median = 6.571) and ER+/PR+ ($n = 8$, median = 3.528). By contrast, *SRA-Del* levels strongly correlated (Spearman coefficient $r = 0.530$, $p = 0.004$) with Nottingham grade scores within the whole cohort ($n = 27$). The level of expression of *SRA* was significantly higher (Mann-Whitney rank sum test, two sided, $p < 0.05$) in tumors with high grade ($n = 7$, median = 6.572) than in tumors with low ($n = 4$, median = 2.192) or intermediate ($n = 9$, median = 2.588) grade (Fig. 4B).

Discussion

Using primers annealing with the core region of the three previously described *SRA* isoforms (9) we have investigated *SRA* expression in 27 independent breast tumors by means of semi-quantitative RT-PCR. These *SRA* isoforms, although different in their 5'- and 3'-terminal region, are all able to co-activate steroid receptor. Indeed, *SRA* core region was found to be necessary and sufficient for the co-activation properties of *SRA* isoforms (9). Therefore, even though PCR performed using primers spanning the *SRA* core region is likely to recognize several different *SRA*-like molecules, the signal obtained corresponds to molecules which should all have essentially the same function, i.e co-activation of steroid receptors.

The expression of *SRA* did not correlate with ER or PR status when the cohort was considered as a whole. This differs from what has been observed for another co-activator, *AIB1*. Indeed, Anzick et al. first showed that a strong expression of *AIB1*, that resulted from *AIB1* gene amplification, was observed in ER positive but not in ER negative breast cancer cell lines (15). More recently, Bautista et al. reported that *AIB1* gene amplification correlated with ER and PR positivity (16). Our results suggest that the pattern of expression of *SRA* is more complex. Indeed, we found that *SRA* expression could correlate positively or negatively with ER and PR levels, depending on the subgroup considered. The general trend appeared to be that in tumors expressing a low level of one receptor (ER or PR), a positive correlation was found between *SRA* expression and the second receptor (PR or ER). Inversely, in tumors highly expressing one receptor (ER or PR), *SRA* expression negatively correlated with the level of expression of the second receptor (PR or ER). At this stage of the knowledge of *SRA* biological function, the interpretation of such an observation is difficult. Indeed, *SRA* has been shown to be able to co-activate both ER and PR (9). Moreover, progestins are known to decrease the steady states of ER- α mRNA and protein, whereas estrogens increase PR expression (17-18). Therefore, all combinations and cross-talk appear possible. One could speculate that increased levels of *SRA* in ER-/PR+ cases could partially be responsible, by "boosting" the activity of the weakly expressed ER, of the expression of PR in these tumors. Inversely, in the same ER-/PR+ cases, the strong *SRA* expression could be responsible of an increased down-regulation of ER by PR. Our results suggest that *SRA* expression varies from one particular tumor to another. Changes in *SRA* expression can be associated with known prognostic and

predictive factors such as ER and PR in particular tumor subgroups. The question of a direct involvement of *SRA* in the hormonal status changes occurring during breast tumor progression remains opened.

Also of interest is the fact that *SRA* interacts with the activation function 1 (AF-1) of the steroid receptors (9). AF-1 is thought to mediate the agonistic effect of antiestrogens such hydroxytamoxifen (19). This agonistic action of antiestrogens is believed to be involved in part in the mechanisms underlying hormone resistance in breast cancer. One could speculate that the level of *SRA* expression might therefore modulate and predict the response of a given tumor to hormone therapy. This hypothesis appears to be refuted by the observation of similar levels of *SRA* in ER+/PR+ and ER-/PR- tumors. But ER+/PR+ tumors, as opposed to ER-/PR- tumors, are likely to respond to endocrine therapy and prevention (see 2 and references herein). In these cases, the differences in ER levels rather than in *SRA* expression are more likely involved in the mechanisms underlying endocrine sensitivity. On the other hand, the observation of a higher *SRA* expression within ER-/PR+, more likely to respond to hormone therapy than ER-/PR- tumors (see 20 and references herein), would be consistent with the hypothesis of a possible involvement of *SRA* in these mechanisms under some circumstances. One should also note that Berns et al. recently reported that even though no correlation was found between the expression of *SRC-1* and ER status, a high expression of this co-activator indicated a favorable response to tamoxifen of patients with recurrent breast cancer (21). This issue can only be addressed in studies performed on tumors from patients that responded or not to endocrine therapy.

We have identified in breast tumor cases a new *SRA* isoform deleted in sequences from nucleotide 155 to 357 (*SRA-Del*). Interestingly, sequence comparison using the BLAST algorithm and the "human EST" database showed that this deleted *SRA* isoform has already been found in a pooled cDNA library containing cDNAs from melanocyte, fetal heart and pregnant uterus (Genbank accession number AA426601). As uterus is another steroid target tissue, it could be hypothesized that the source of *SRA-Del* in this pooled library was indeed uterus. Even though the structure of the *SRA* gene has not yet been published, *SRA-Del* appears to correspond to a perfect exon-3 deleted *SRA* variant. *SRA* gene has recently been located on chromosome 5q31.3-32.⁴ Sequence analysis of the corresponding DNA sequence (chromosome 5, BAC clone 319C17, Genbank accession number AC005214) revealed that the fragment from nucleotide 155 to 357 corresponds to the third *SRA* exon. The putative function of *SRA-Del* remains to be determined. One should however note that a

recombinantly developed *SRA* mutant, deleted of the region 3' of a BbsI site (position 341) and therefore partially deleted of exon-3 sequences, did not co-activate steroid receptors (9). Moreover, exon-3 deletion introduces a shift in the open reading frames suggested by Lanz et al. (9) and could lead to a premature termination of the putative *SRA* proteins. One could therefore hypothesize that *SRA*-Del might interfere with *SRA* activity. The resulting modifications of the steroid receptor signaling pathways could confer a more aggressive behavior to the tumors expressing higher levels of *SRA*-Del. The positive correlation between *SRA*-Del levels and tumor grade scores would be consistent with this hypothesis.

Interestingly, modifications of the long arm of the chromosome 5 have been reported in breast tumors. Indeed, Hermesen et al. (22) found a frequent chromosomal gain in 5q within a subset of 53 lymph node-negative breast carcinoma whereas Schwendel et al. observed a frequent loss of this region in 39 invasive breast carcinomas (23). Moreover, among *BRCA1* mutation carriers, loss of 5q was observed more frequently than in the control patient (24). One could therefore speculate that *SRA* is one of the genes whose loss is selected for during tumor progression in cells lacking *BRCA1* functional gene. Whether changes in *SRA* expression result from chromosomal abnormalities remains to be determined.

In conclusion we have shown that *SRA* is expressed in breast tumors and that its expression correlates with ER and PR levels in particular tumor subgroups. We speculate that changes in *SRA* expression could be involved in the mechanisms underlying tumor progression and hormone resistance.

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Figures legend

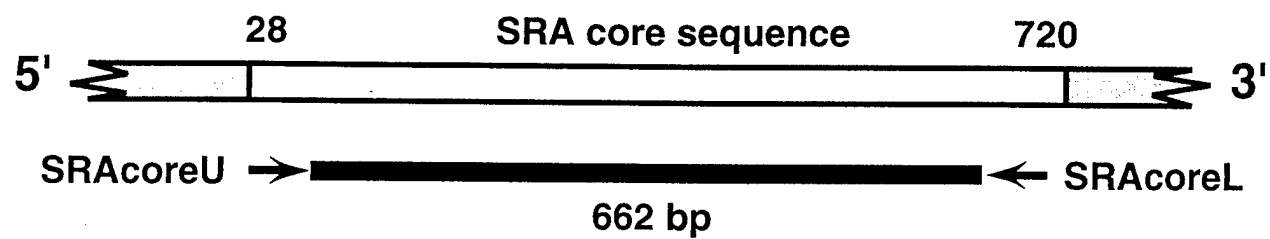
Fig. 1 SRA structure and primers presentation. *SRA* isoforms identified to date (9) differ in their 5' and 3' terminal regions (gray boxes) but present an identical nucleotide sequence (white box) in between (*SRA* core sequence, bases 28 to 720). *SRA*coreU and *SRA*coreL primers anneal with *SRA* core sequences and allow the amplification of a 662 bp long fragment.

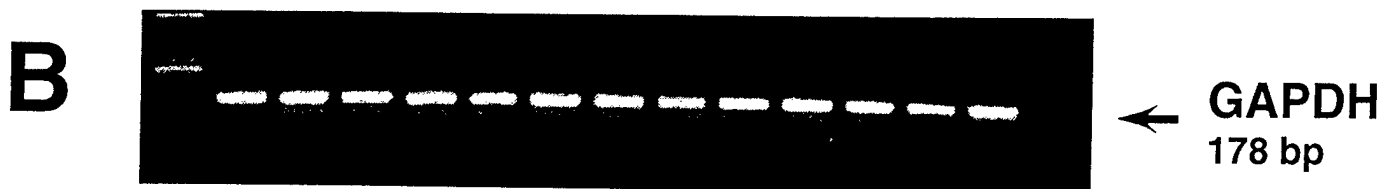
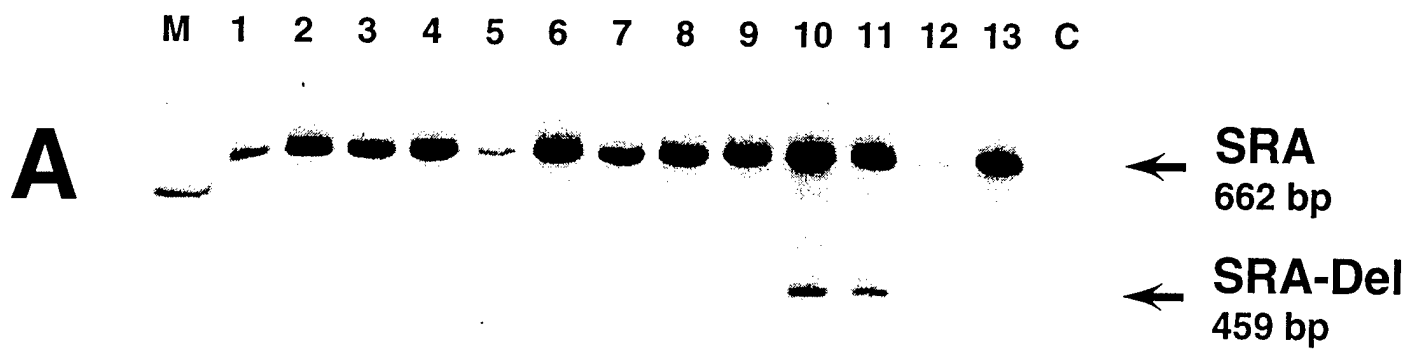
Fig. 2 Detection of *SRA* in human breast tumors by RT-PCR. Total RNA was extracted from 27 breast tumors, reverse transcribed and analyzed by PCR as described in the Materials and Methods section. PCR products were separated on 6% acrylamide gels. Gels were dried and exposed 1 h to a Molecular ImagerTM-FX Imaging screen. Screens were then scanned using a Molecular ImagerTM-FX. **A.** computerized image showing the results obtained for 13 cases (1-13). M: molecular weight marker (ϕ x174 RF DNA/Hae III fragments). C: control lane, no cDNA added in the PCR. Sequencing analysis of PCR fragments revealed that the 662 bp (*SRA*) and 459 bp (*SRA*-Del) long fragment corresponded to *SRA* and to a variant *SRA* isoform deleted in sequences from position 155 to 357 (Genbank accession number AF092038), respectively. **B.** ethidium bromide stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples.

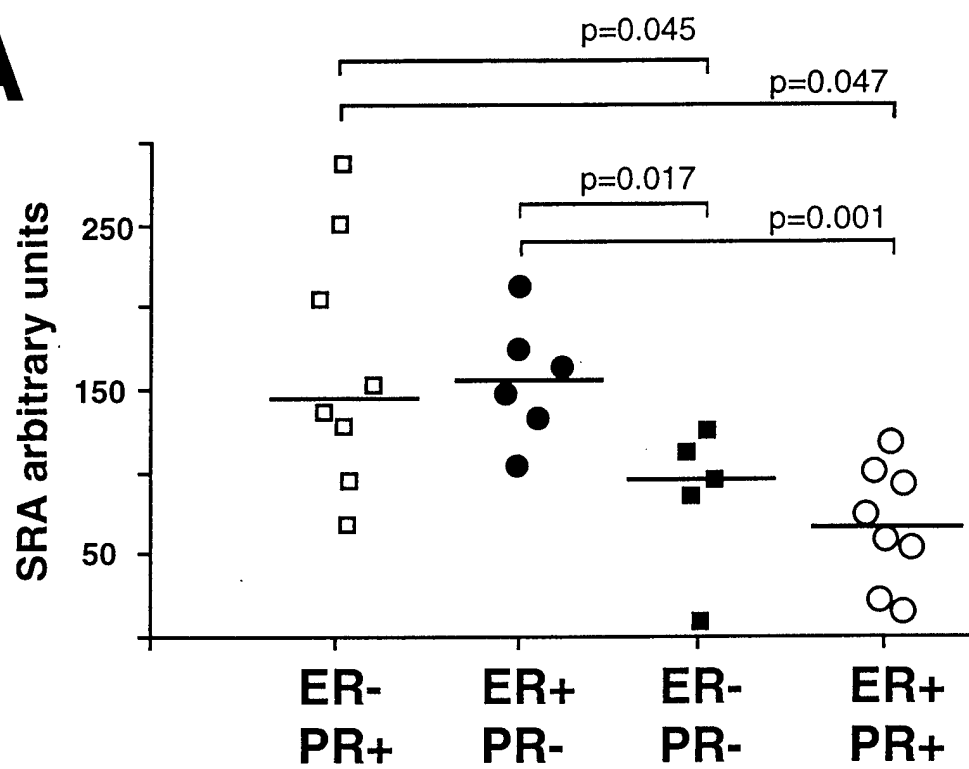
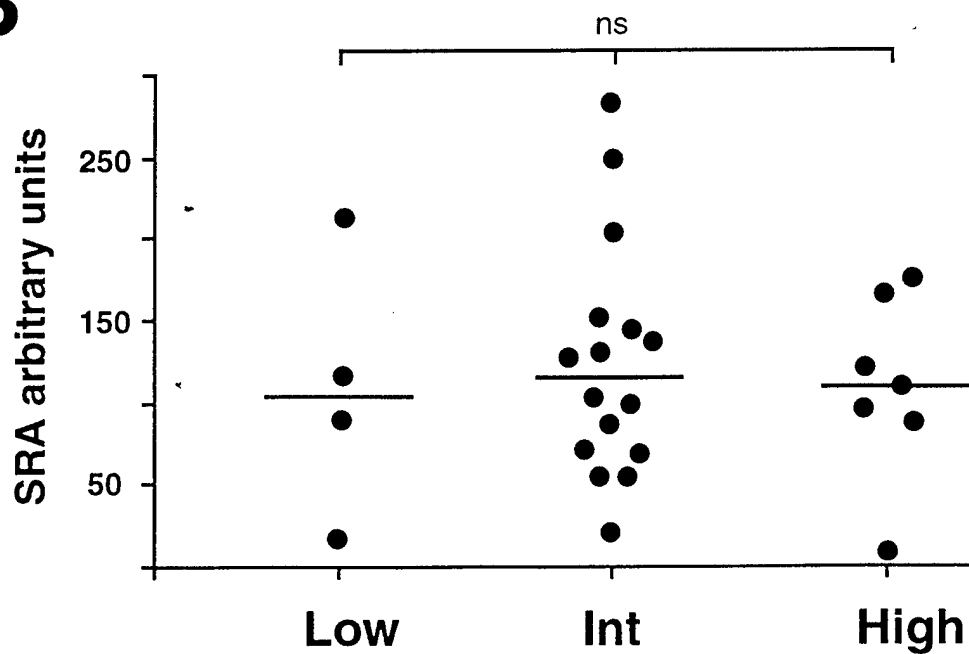
Fig. 3 Subgroup analysis of *SRA* expression within 27 human breast tumors. For each case, *SRA* expression was quantified and expressed in arbitrary units as described in the "Materials and Methods" section. **A.** Tumors were grouped according to their ER and PR status, as determined by ligand binding assay. ER-/PR+ tumors are open squares; -ER+/PR- tumors are black circles; ER-/PR- tumors are black squares; ER+/PR+ tumors are open circles. **B.** Tumors were grouped according to their grade: low (Nottingham grading scores 4-5), intermediate (Nottingham grading scores 6-7) and high (Nottingham grading scores 8-9). The horizontal line represents the median value in each group. p values (Mann-Whitney rank sum test, two-sided) were indicated when subgroups are statistically different. ns: no statistically significant differences were found between subgroups.

Fig. 4 Subgroup analysis of *SRA-Del* relative expression within 27 human breast tumors. For each case, *SRA-D3* expression relative to *SRA* was quantified as described in the "Materials and Methods" section.

A. Tumors were grouped according to their ER and PR status, as determined by ligand binding assay. ER-/PR+ tumors are open squares; ER+/PR- tumors are black circles; ER-/PR- tumors are black squares; ER+/PR+ tumors are open circles. **B.** Tumors were grouped according to their grade: low (Nottingham grading scores 4-5), intermediate (Nottingham grading scores 6-7) and high (Nottingham grading scores 8-9). The horizontal line represents the median value in each group. p values (Mann-Whitney rank sum test, two-sided) were indicated when subgroups are statistically different. ns: no statistically significant differences were found between subgroups.





A**B**

**Increased Expression of the Steroid Receptor RNA Activator (SRA)
During Human Breast Tumorigenesis.**

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APPENDIX 18

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Increased expression of the steroid receptor RNA activator
during human breast tumorigenesis.

Submitted, Cancer Res.

Abstract.

Using reverse transcription polymerase chain reaction assays, expression of SRA was compared between adjacent normal human breast tissue and matched breast tumors from 19 patients. Core SRA RNA was detected in normal and neoplastic breast tissues. The level of SRA RNA was significantly ($p = 0.0004$) higher in breast tumors than in matched normal breast. A deleted SRA RNA was detected in most samples of normal breast and tumors. No differences occurred in the relative expression of the deleted SRA between normal breast and tumors. Within the breast tumor cohort the relative expression of the deleted SRA was positively correlated with tumor grade ($r = 0.556$, $p = 0.0135$) and size ($r = 0.655$, $p = 0.0023$). These data suggest that expression of core SRA is upregulated during breast tumorigenesis and that changes in the relative expression of a deleted SRA isoform occur during breast cancer progression.

Introduction.

Recently, a novel steroid receptor coactivator, SRA (1) was isolated and characterized. Interestingly, the available data suggest that the SRA RNA transcript is the functionally important molecule with respect to its steroid receptor activator activity (1) and is found within ribonucleoprotein complexes which may also contain other steroid receptor activators such as SRC-1 (2) which are functional as proteins. Previously, we have identified the expression of SRA RNA in human breast tumor biopsy samples (3). In contrast to another steroid receptor activator, AIB1, whose overexpression is correlated with estrogen (ER α) and progesterone receptor (PR) expression (4) SRA expression was not correlated overall with steroid receptor status as measured by ligand binding assays (3). Although the expression of a deleted form of SRA RNA was found to correlate with increasing tumor grade, the total expression of SRA-like RNA was unrelated to markers of progression and endocrine sensitivity in human breast tumors overall (3). These previous results, however, did not address the possibility that SRA expression could be altered during breast tumorigenesis. Since estrogen receptor signaling is thought to be altered during breast tumorigenesis as well as breast cancer progression, we have tested the hypothesis that altered expression of SRA is associated with breast tumorigenesis and may contribute to altered estrogen signaling during breast tumorigenesis.

Materials and Methods.

Human Breast Tissues.

Nineteen ER positive primary human breast tumor biopsies (ER positivity defined as > 3 fmol/mg protein in classical ligand binding assays) were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The ER levels ranged from 3.7 - 83 fmol/mg protein and the PR levels ranged from 2.7 - 112 fmol/mg protein (PR positivity defined as > 10 fmol/mg protein in classical ligand binding assays; 14 tumors were PR+ and 5 tumors were PR -). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block and the relative cellular composition was determined by the histopathological assessment of sections from adjacent mirror image paraffin-embedded tissue blocks, as previously described (5). The presence of normal ducts and lobules as well as the absence of any atypical lesion was confirmed in all normal tissue specimens. The tumors spanned a wide range of grades (grade scores 5-9) as determined by the Nottingham grading system (6), and ranged in size from 0.6 - 6.4 cm.

RNA Extraction and RT-PCR conditions.

Total RNA was extracted from 20 μ m frozen tissue sections (20 sections per tumor; 35 sections for normal tissues) using Trizol™ reagent (Life Technologies, NY) according to the manufacturer's instructions and quantified spectrophotometrically. One μ g of total RNA was reverse transcribed in a final volume of 25 μ l as previously described (7).

Primers and PCR conditions.

The primers used were SRAcoreU primer (5'-AGGAACGCGGCTGGAACGA -3'; sense; positions 35-53, Genbank accession number AF092038) and SRAcoreL primer (5'- AGTCTGGGGAACCGAGGAT -3'; antisense; position 696-678, Genbank accession number AF092038). PCR amplifications were performed and PCR products analyzed as previously described (7) with minor modifications. Briefly, 1 μ l of reverse transcription mixture was amplified in a final volume of 15 μ l, in the presence of 1.5 μ Ci of (α -³²P) dCTP (3000 Ci/mmol), 4 ng/ μ l of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY). Each PCR consisted of 30 cycles (30 sec at 60°C, 30 sec at 72°C and 30 sec at 94°C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and exposed 2 hours to a Molecular ImagerTM-FX Imaging screen (Bio-Rad, Hercules, CA). Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as previously described (7). Identity of PCR products was confirmed by subcloning and sequencing, as previously reported (8).

Quantification of SRA expression.

Exposed screens were scanned using a Molecular ImagerTM-FX (Bio-Rad, Hercules, CA) and the intensity of the signal corresponding to SRA was measured using Quantity OneTM software (Bio-Rad, Hercules, CA). Three independent PCRs were performed. In order to control for variations between experiments, a value of 100% was arbitrarily assigned to the SRA signal of one particular tumor (tumor #14) measured in each set of PCR experiments

and all signals were expressed as a percentage of this signal. In parallel, *GAPDH* cDNA was amplified and following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst™ (Bio-Rad, Hercules, CA). Three independent PCRs were performed. Each *GAPDH* signal was also expressed as a percentage of the signal observed in the tumor #14. For each sample, the average of SRA signal was then expressed as a percentage of the *GAPDH* signal (arbitrary units).

Quantification of the relative expression of the deleted SRA variant RNA.

It has previously been shown that the co-amplification of a wild-type and a deleted variant cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (8, 9). For each sample, SRA_{del} corresponding signal was measured using Quantity One™ software (Bio-Rad, Hercules, CA) and expressed as a percentage of the corresponding core SRA signal. For each case, 3 independent assays were performed and the mean determined.

Statistical analysis

Differences between normal samples and their matched tumors were tested using the Wilcoxon matched pairs test, two-tailed. Correlation between SRA expression and tumor characteristics was tested by calculation of the Spearman coefficient r .

Results.

Detection of SRA and a deletion variant RNA form in both normal and neoplastic human breast tissues.

Using the SRA specific primers described in the "Materials and Methods" section, which amplify the core SRA sequences (Figure 1) as described by Lanz *et al.*, (1), we have previously detected two PCR products of 662 bp and 459 bp in human breast tumors (3). Cloning and sequencing revealed the identity of the 662 bp fragment with the SRA core region (1), and the 459 bp fragment as a variant form of SRA deleted in 203 bp between positions 155 and 357 (Figure 1, as numbered according to Genbank accession number AF092038). The current analysis identified the 662 bp product in all breast tissue samples assayed, both normal and neoplastic. As well, a 459 bp product corresponding to a SRA transcript deleted in 203 nucleotides was detected in the majority of tumors ($n = 18$) and normal samples ($n = 17$), and always together with the 662 bp product (Figure 2). Therefore, SRA core sequences are expressed in all human breast tissues and the expression of the deleted SRA transcript is not tumor specific.

Comparison of the expression of SRA and deleted SRA in adjacent normal breast tissue and matched primary breast tumors.

To determine whether alterations in core SRA expression occur during breast tumorigenesis, the expression of SRA RNA was measured in primary tumor tissues and their adjacent matched normal breast tissues from 19 different patients, as described in the Materials and Methods section. The analysis was confined to tissues from women whose breast tumor was ER+, as determined by ligand binding assays. Examples of the results obtained are shown in Figure 2. The expression of SRA corrected for the *GAPDH* signal in

each tissue sample for all the matched normal and tumor pairs is shown as a scatter graph in Figure 3A. The level of expression of core SRA was found to be significantly higher (Wilcoxon matched pairs test, $p = 0.0004$) in the tumor samples compared to their adjacent normal breast tissue. When the deleted SRA is detected, the expression of the variant SRA transcript relative to the core SRA expression was not significantly different between normal breast tissues and their matched adjacent breast tumors (Figure 3B). These data suggest that core SRA expression is upregulated during breast tumorigenesis, but the relative expression of a deleted SRA variant is not altered during breast tumorigenesis.

Correlation of SRA expression and the relative expression of deleted SRA with tumor characteristics.

The level of core SRA expression in the tumor cohort used in this study was not correlated with PR status, grade, tumor size or nodal status. However, the relative expression of the deleted SRA transcript in the tumors was positively correlated with grade score (Spearman $r = 0.556$, $p = 0.0135$), and tumor size (Spearman $r = 0.655$, $p = 0.0023$) but was not correlated with PR status or nodal status. These data suggest that increased relative expression of a deleted SRA variant is more likely to occur in those breast tumors with characteristics of a poorer prognosis, and may be associated with breast tumor progression.

Discussion.

SRA is a novel steroid hormone receptor transcriptional activator. In particular it is different from other steroid receptor activators in three important ways: firstly it functions as a RNA molecule rather than being dependent on translation into a protein; secondly, it is specific for steroid hormone receptors; and thirdly, rather than effecting AF2 of steroid hormone receptors, it is specific

for the AF1 domain of these receptors (1). Since steroid hormones, in particular estrogen, play an important role(s) in the growth and function of both the normal and neoplastic human breast (10, 11) and since altered estrogen receptor signaling likely occurs both in breast tumorigenesis and breast cancer progression (12-15), alteration of factors which may influence ER activity during breast tumorigenesis and/or progression are relevant to investigate in human breast tumorigenesis *in vivo* (16, 17). Moreover, there are data which support increased expression of at least one steroid receptor activator, AIB1, in human breast cancers *in vivo* (18).

The data presented suggest that the expression of the novel steroid receptor activator, SRA, is significantly increased in ER positive human breast tumors *in vivo* compared to their adjacent matched normal breast tissue. Since SRA is functional as a steroid receptor activator as a RNA molecule (1), measurement of the level of SRA RNA likely reflects functional equivalents of the molecule in the breast tissues, and therefore our data support the hypothesis that the activity of this receptor activator is significantly upregulated during human breast tumorigenesis and therefore may have a role in increasing estrogen receptor signal transduction and sensitivity during breast tumorigenesis. Interestingly, our previous data (3) suggest that the level of expression of SRA in primary tumors, in contrast to AIB1 (4), is not correlated with ER and PR status overall, which suggests that SRA may subserve other functions in ER and PR negative tumors compared to ER and PR positive tumors. However, subgroup analysis within a previous cohort (3) showed that SRA expression correlated with either ER or PR depending on the subgroup considered. The general trend was that in tumors with a low level of expression of one receptor either ER or PR, a positive correlation was found between SRA and the second receptor, either PR or ER. By contrast in tumors

expressing a high level of one receptor, either ER or PR, a negative correlation of SRA expression with the level of expression of the second receptor, either PR or ER was found. The functional significance of such correlations is unclear at this stage, but may reflect compensatory regulation under conditions of potentially reduced steroid responsiveness.

While we cannot exclude the possibility that the differences seen between normal and breast tumor tissue are due to the different levels of cellularity between the tissues, we have found no correlations of SRA expression with different percentages of epithelial and stromal cell types in either the normal or the tumor compartments of this cohort (data not shown). Previously, SRA was shown to be expressed in human breast cancer cell lines in culture (1), consistent with the idea that SRA is expressed in epithelial cells. Our data are consistent with this observation and together the data support the hypothesis that the expression of SRA is increased significantly in human breast cancer cells both *in vivo* and *in culture*.

At least three isoforms of SRA RNA have been reported (1), containing the so-called core region (see Figure1) but differing in the regions outside of the core region, both 5' and 3'. It is the core region of SRA which is necessary and sufficient for the steroid receptor activator activity of SRA. The design of our primers for SRA as previously described (3), will detect all SRA isoforms containing core sequences. In the absence of data characterizing potential individual functions of SRA isoforms differing in the 5' and 3' regions outside of an intact core region, we assume that our measurement of all intact core SRA like RNAs correlates with the total SRA activity present in any one tissue sample. These primers, however, also detect a distinct but previously described isoform of SRA (Genbank accession number AA426601) containing a deletion of sequences within the SRA core region. Deletions within the core

region were previously reported to result in loss of SRA activator function (1). Although the specific deletion found in the naturally occurring SRA deleted variant RNA has not been characterized per se, the currently published data suggest that it is likely that this deleted variant is inactive with respect to steroid receptor activator activity. It could therefore function to alter steroid signaling pathways in human breast tumors and may contribute to the more aggressive phenotype associated with poorer prognosis breast tumors, which include characteristics such as high grade and large tumor size.

In conclusion our data support a significant upregulation of total core SRA-like expression and likely activity during breast tumorigenesis. This upregulation, in combination with that of other steroid receptor activators and increased expression of ER α are all likely to contribute to enhanced estrogen signaling pathways which occur during and possibly contribute to human breast tumorigenesis.

Acknowledgements.

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Legends to Figures.

Figure 1.

Primers and expected PCR products for core SRA and deleted SRA.

SRAcoreU and SRAcoreL primers anneal with SRA core sequences (white boxes, bases 28-720, see reference 1) and allow the amplification of a 662 bp and a 459 bp long fragment corresponding to the wild-type SRA core mRNA and a SRA mRNA deleted from bases 155 to 357 (SRA_{del}, see reference 3), respectively. The 5' and 3' variable regions of the different SRA isoforms previously identified (1) are indicated by gray boxes.

Figure 2.

Detection of SRA in normal breast tissue adjacent to matched primary, ER+ invasive breast cancer. RNA extracted from matched breast tumors and adjacent matched normal breast tissue was extracted from 19 different patients and assayed for SRA expression using RT-PCR as described in Materials and Methods. PCR products were separated on 6% acrylamide gels, which were dried, exposed to phosphor-imaging screens, and scanned using a Molecular ImagerTM-FX. **A.** A digitized image showing the results obtained from 4 sets of normal tissue (N) and matched tumor tissue (T) is shown. The arrows identify the expected 662 bp core SRA PCR product (SRA core, confirmed by sequence analysis) and a 459 bp deleted SRA variant PCR product (SRA_{del}), which was identified by sequence analysis to correspond to an SRA variant deleted in sequences from position 155 to 357 (Genbank accession number AF092038). **B.** Ethidium bromide stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples. The arrow identifies the expected 178 bp *GAPDH* PCR product.

Figure 3.

A. Comparison of the expression of SRA in adjacent normal breast tissue and matched primary breast tumors. For each patient ($n = 19$), SRA expression was quantified and expressed in arbitrary units corrected for *GAPDH* signal as described in the Materials and Methods. The results are presented as a scatter graph. The normal samples are represented by open squares and the tumor samples by filled squares. Each matched normal and tumor sample is joined by a line. The level of SRA expression in normal tissue is significantly different to the level of SRA expression in the tumor tissues (Wilcoxon matched pairs test, two-tailed, $p = 0.0004$).

B. Comparison of the relative expression of the deleted SRA variant in adjacent normal breast tissue and matched primary breast tumors. For each sample, the signal corresponding to the deleted SRA variant RNA (SRA_{del}) was measured using Quantity One™ software (Bio-Rad, Hercules, CA) and expressed as a percentage of the corresponding core SRA signal as described in the Materials and Methods. The results are presented as a scatter graph. The normal samples are represented by open squares and the tumor samples by filled squares. Each matched normal and tumor sample is joined by a line. There is no significant difference between the relative expression of SRA_{del} in normal samples and their matched adjacent tumors.

Figure 1.

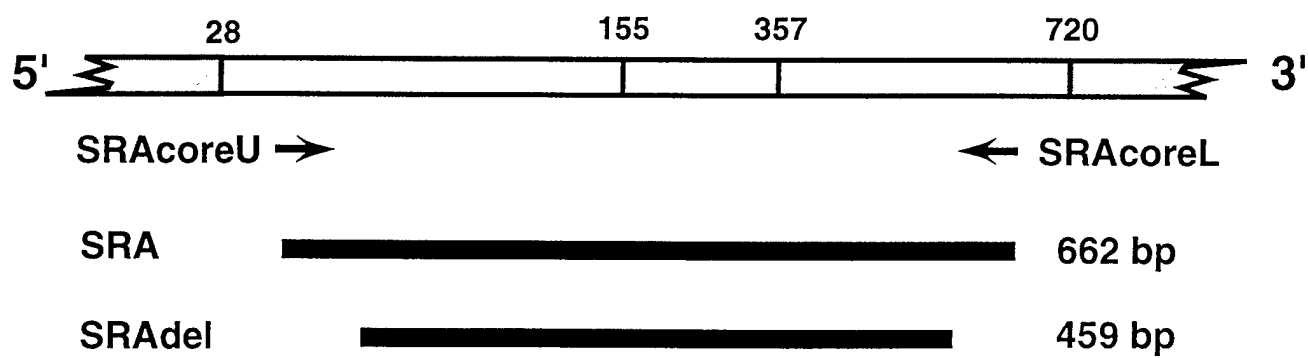
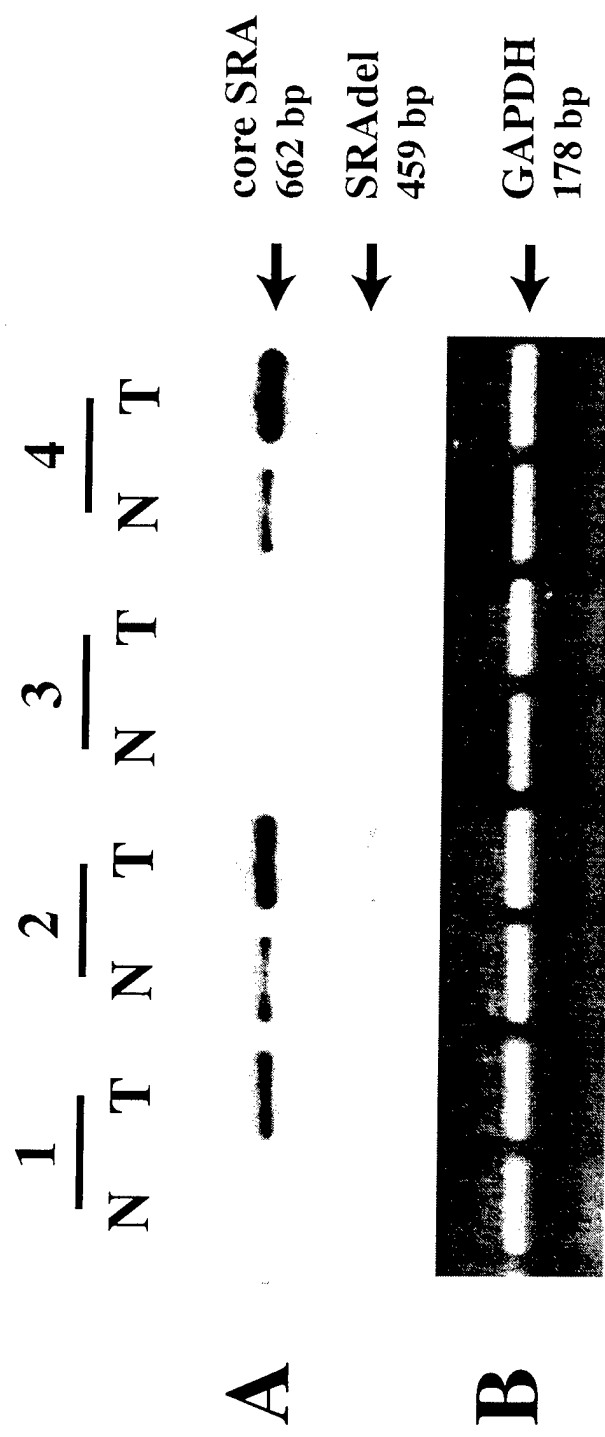


Figure 2



APPENDIX 19

Murphy LC, Dotzlaw H, **Leygue E**, Douglas D, Coutts A, and Watson PH

Estrogen receptor variants and mutations.

J Steroid Biochem Mol Biol, 62: 363-372, 1997.



MiniReview

Estrogen Receptor Variants and Mutations

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There is a large and increasing body of experimental and clinical data supporting the existence of variant estrogen receptor (ER) proteins in both normal and neoplastic estrogen target tissues, including human breast. Therefore, future examination of ER signal transduction and/or measurement of ER protein must take into account variant ER expression. The functions of variant ER proteins, either physiological or pathological, remain unclear, although a role(s) for some ER variants in breast tumorigenesis and breast cancer progression would be consistent with the accumulated data. Possible tissue specific expression leads to the speculation that ER variants may have a role in tissue specific estrogen action. The following review focuses on the current knowledge available in the scientific literature with respect to the type and characteristics of estrogen receptor variants and mutations that have been identified to occur naturally in tissues and cell lines. © 1997 Published by Elsevier Science Ltd. All rights reserved

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INTRODUCTION

Estrogens are major regulators of many physiological functions, especially those associated with reproduction and mammary gland development [1]. As well, estrogens are involved in the growth and development of both uterine and mammary cancers [2]. The principal mechanism by which the effects of estrogen are mediated in either normal or neoplastic target cells is via an initial interaction with the estrogen receptor (ER). The ER is a member of the steroid/thyroid/retinoid receptor gene superfamily [3]. The protein products of this family are intracellular ligand-activated transcription factors regulating the expression of several gene products, which ultimately result in a target tissue specific response. Ligands for the ER include steroidal and nonsteroidal estrogens and antiestrogens [4]. Furthermore, ligand-independent activation of the ER can occur in both normal and neoplastic target cells [5, 6].

The effects of estrogen and antiestrogens on various target tissues can be markedly different even at the level of regulation of expression of the same gene in different tissues or cell types [7–10]. Furthermore, it is thought that perturbations of the ER signal transduction pathway are likely to contribute to tumor progression, especially in mammary cancer and the eventual development of a hormone-independent and more aggressive phenotype [11]. One mechanism underlying tissue specific differences and altered responsiveness associated with cancer development and progression could be alterations in the structure and therefore function of the ER itself. This review will focus on structural changes in the ER that have been identified to occur naturally in tissues and cell lines.

A LARGE BODY OF MOLECULAR EVIDENCE EXISTS TO SUPPORT THE EXPRESSION OF VARIANT AND MUTANT ER MRNA SPECIES

ER-like mRNAs distinct from the wild-type ER mRNA have been identified in many known ER posi-

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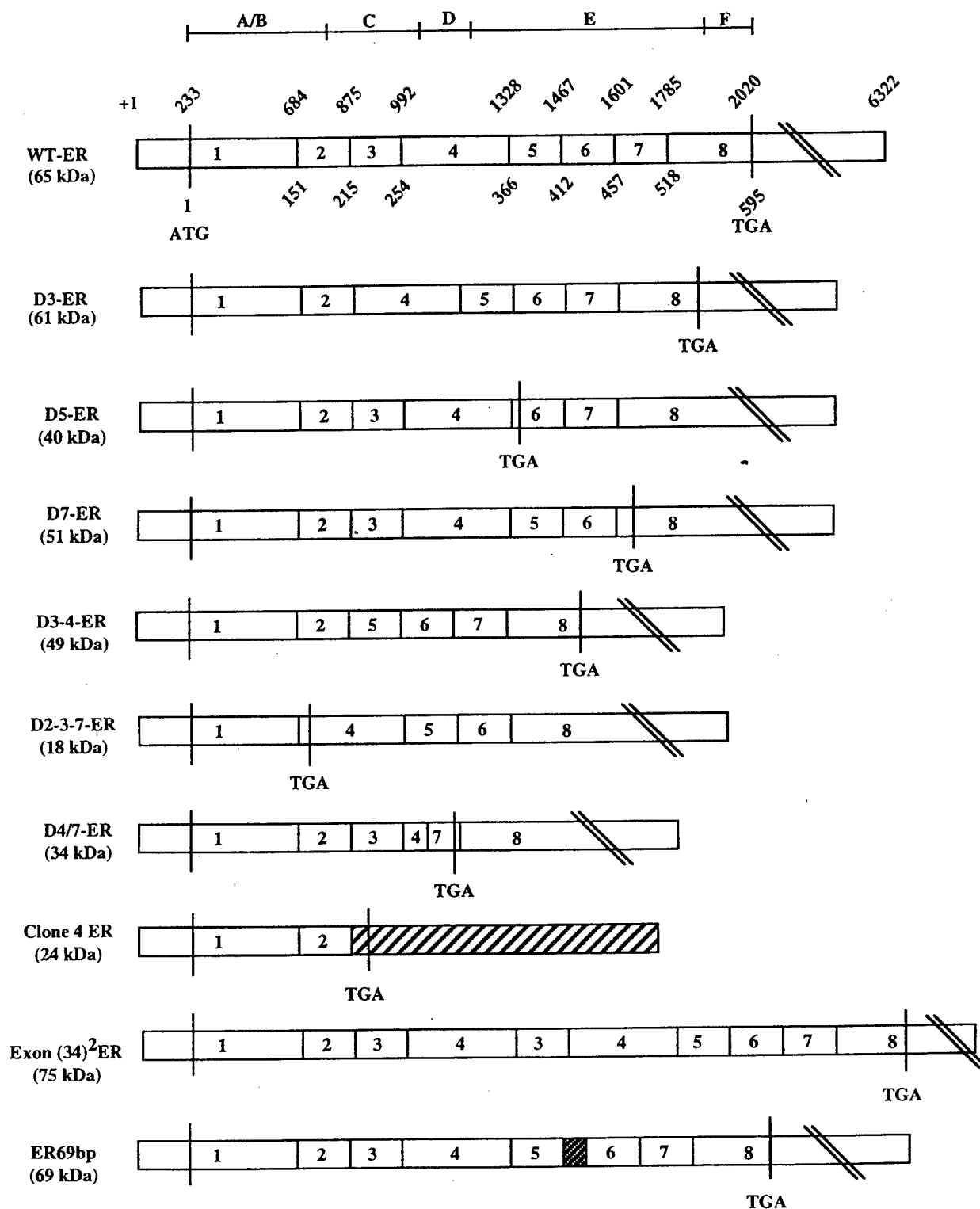


Fig. 1. Schematic diagram of the wild-type human estrogen receptor (ER) cDNA, which contains 8 different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in the trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another trans-activating function (AF-2). The numbering on the top of the cDNA refers to the nucleotide position as defined in Ref. [69]. Below the wild-type ER cDNA are the various putative exon and other large deleted ER cDNAs. ATG shows the translation initiation codons, TGA shows the inframe translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in Ref. [69]. D = deletion and the estimated molecular mass (kDa = kiloDaltons) of each open reading frame is shown in brackets. Molecular masses were estimated using MacVector version 4.1.4 software.

tive tissues and cell lines. Several different patterns of ER-like transcripts have been characterized. A few of these have been cloned and characterized from cDNAs representing close to full-length transcripts, but in most cases their overall structure has been predicted from more limited sequences derived from reverse transcribed and polymerase chain reaction amplified (RT-PCR) products. By virtue of specific primer design this experimental approach has focused attention on small regions of the known wild-type ER mRNA.

Transcripts containing precise single or multiple exon deletions

Multiple ER-like transcripts have been identified which contain precise exon deletions [12–21]. Examples of exon deleted transcripts described in the literature are shown in Fig. 1. More recently, however, ER-like transcripts containing two or three entire exon deletions have been detected in cell lines and tissue samples [17–21]. Perhaps more importantly, the identification of multiple types of exon deleted transcripts in any one cell line or tissue sample [17–19,21] underscores the need to study

these variant ER transcripts altogether, as well as individually.

Other deleted transcripts

ER-like transcripts containing variable sized deletions which are not entire exon deletions have also been detected. This type of alteration falls into two groups: one in which a single nucleotide has been deleted [22,23] and the other in which several hundreds of continuous nucleotides have been deleted but starting and ending within known exon sequences (Fig. 1, Table 1) [21,22,24].

Truncated transcripts

These altered ER-like transcripts are significantly smaller than the wild-type ER mRNA as determined by Northern blot analysis [26,27]. cDNA cloning was used to characterize these transcripts. Such studies identified two types of truncated transcripts: those that are truncated from the 3' end compared to the wild-type ER mRNA [28] and those that are truncated from the 5' end compared to the wild-type ER mRNA [27].

The 3' truncated transcripts (characterized from apparently close to full-length cDNA clones) contain

Table 1. ER variants identified in tissues and cell lines

Variant mRNA	Estimated M_r of predicted protein (kDa)	Functional domains	Tissue/cell line	Refs.
Wild-type ER	65	A, B, C, D, E, F	N, T targets	[69,80,81]
D2-ER	16	A, B?	N, T breast	[12,17–20]
D3-ER	61	A, B, D, E, F	N, T breast	[12,15]
D4-ER	54	A, B, E?, F	N, T breast, ovary, rat bone uterus; M, N rat brain	[13,18–20,25] [39,48,57,63,79]
D5-ER	40	A, B, C, D	N, T breast, uterus HC, PBMC	[14,25,58–60]
D6-ER	52	A, B, C, D	T breast	[15]
D7-ER	51	A, B, C, D	N, T breast, uterus	[12,16,21,25]
D2-3-ER	18	A, B?	N, T breast	[17,21]
D3-4-ER	49	A, B, E?, F	N, T breast, rat bone, uterus VSMC	[17,18,21,25,79] [47]
D4-5-ER	28	A, B, C?	VSMC; N, T uterus	[25,47]
D4-7-ER	39	A, B	N, T, breast T breast cells TamR	[19–21] [20]
D2-3-4-ER	17	A, B?	T breast	[21]
D2-3-7-ER	18	A, B?	T breast	[21]
D3-4-5-ER	24	A, B	N, T breast	[19]
D5-6-7-ER	41	A, B, C	N, T breast	[19]
D3/7-ER	27	A, B	T breast	[21]
D4/5-ER	49	A, B, C?, F	T breast cells	[22]
D4/7-ER	34	A, B, C?	N uterus, T breast	[24]
clone 4-ER	24	A, B	N, T breast	[28]
clone 24-ER	37	A, B	breast tumor	[28]
TERP-1	20	?E, F	female rat pituitary	[27]
TERP-2	19	?E, F	female rat pituitary	[27]
exon 6 ² -ER	51	A, B, C, D	breast tumor	[29]
exon (34) ² ER	75	A, B, C + , D + , E, F	breast tumor	[29]
exon (67) ² ER	80	A, B, C, D, E + , F	MCF-7 subline	[30,36]
ER69bp	69	A, B, C, D, E?, F	breast tumor	[29,31]

M = meningiomas; HC = hepatocarcinoma; PBMC = peripheral blood mononuclear cells; VSMC = vascular smooth muscle cells; N = normal; T = tumorous; Tam^R = tamoxifen resistant.

entire exon sequences of at least 2 of the 5' ER exon sequences and then diverge into ER unrelated sequences [28], some of which appear to be LINE-1 related (Fig. 1, Table 1). Although several different truncated ER mRNAs have been cloned, some of these were only found to be expressed in a single breast tumour, although others such as the clone 4 truncated ER mRNA have been found to be commonly expressed in human breast tumours [28].

The 5' truncated transcripts have been identified and characterized so far only in normal rat pituitaries [27]. From oligonucleotide hybridization and anchored PCR analyses these transcripts were found not to contain exons 1–4 of the wild type rat ER mRNA, but contain ER unrelated sequences at their 5' end followed by sequences found in exons 5–8 of the wild type rat ER mRNA. Two similar but slightly different transcripts (named TERP-1 and TERP-2) of this type have been characterized [27].

Insertions

ER-like transcripts have been identified containing variable sized nucleotide insertions. Such insertions consist of one to two nucleotides [22, 23], larger insertions of 69 and more nucleotides [22, 29] and apparently complete exon duplications [29, 30] (Fig. 1, Table 1). As well an ER-like transcript containing a novel 16 nucleotide insertion in exon 8 combined with a partial deletion in this exon was identified in a human uterine tumor samples [25]. These abnormal ER-like transcripts were detected using RT-PCR analyses, and further studies showed that the exon 6 plus 7 duplicated ER-like transcript was generated from a

mutated ER gene in which genomic rearrangement resulted in the duplication of exons 6 and 7 in an in-frame fashion [30]. As well the 69 bp inserted ER mRNA is likely generated from a point mutation in one allele of the ER gene in the breast tumour from which it was cloned. This point mutation generates a consensus splice donor site at the 3' end of the 69 bp sequence present normally in intron 5. As well a splice acceptor consensus sequence is normally present at the 5' end of the 69 bp sequence and thus the 69 sequences are likely to be seen as another exon in the gene [31].

Point mutations

Several point mutations including silent polymorphisms have been identified in ER-like transcripts (Table 2) [23, 28, 66–68]. The only known germline mutation in the human ER associated with disease is a point mutation [33], identified in a young adult male presenting with osteoporosis, unfused epiphyses, continued linear growth in adulthood, and estrogen resistance. Furthermore, only approximately 1% of primary breast tumors have point mutations in the ER gene [23, 81], which in some cases might be linked to hereditary breast cancer [34].

The above ER-like mRNA molecules have most often been identified in human breast cancer tissues or cell lines. However, there is now emerging data showing that several of the exon deleted and truncated transcripts are also expressed in normal human breast tissue [17–19]. Furthermore, these variant ER transcripts are always found co-expressed with the wild-type ER mRNA, with little evidence of accompa-

Table 2. Small insertions/deletions and point mutations/polymorphisms identified in the estrogen receptor mRNA

Nucleotide change	Exon	Amino acid change	Functional domains	Ref.
262 T → C	1	10 Ser no change	A, B, C, D, E, F	[28], Ref. 81
439 C → G	1	69 Asn → Lys	A, B?, C, D, E, F	Ref. 81
493 G → C	1	87 Ala no change (B variant)	A, B, C, D, E, F	[66–68], Ref. 81
701 C → T	2	+Stop after 156	A, B?	[33]
961 C → T	3	243 Arg no change	A, B, C, D, E, F	Ref. 81
TT insert after 981	3	Met 250 → Ile + stop	A, B	[22]
1059 C → T	4	276 Gly no change	A, B, C, D, E, F	[23]
1119 T → C	4	296 Leu → Pro	A, B, C, D?, E?, F	[68]
1207 C → G	4	325 Pro no change	A, B, C, D, E, F	[68], Ref. 81
1283 G → T	4	352 Asp → Try	A, B, C, D, E?, F	[55]
1290 A → T	4	353 Glu → Val	A, B, C, D?, E?, F	[23]
1418 A → G	5	396 Met → Val	A, B, C, D, E?, F	Ref. 81
1463 G del	5	411 Asp → Thr	A, B, C, D	[22]
		+6 extra novel a.a.		
del T at 1526	5	432 Ser → His + 4 extra novel a.a.	A, B, C, D	[23]
		424 Ile → Arg +28 extra novel a.a.	A, B, C, D	[23]
1503–1550 replaced by 1380–1422	6			
1647 G → A	7	472 Lys no change	A, B, C, D, E, F	[23]
1747 C → G	7	505 Ala no change	A, B, C, D, E, F	[23]
1963 T → C	8	577 His no change	A, B, C, D, E, F	[23]
2014 A → G	8	594 Thr no change	A, B, C, D, E, F	Ref. 81

Nucleotides are numbered according to the start site of transcription (+1) in Ref. [69]. ? = an alteration of the function has been shown or is likely to occur.

nying gross deletions or rearrangements of the ER gene in the tissues or cell lines [26,32]. This suggests that the mechanisms for generating these transcripts are present in normal human mammary cells and therefore these transcripts are normal variants, and are likely to have been generated by an alternative splicing mechanism [32]. Furthermore, many ER-like mRNAs have been identified in other normal and neoplastic tissues as detailed in Table 1. It is less likely that the inserted transcripts and many of the amino acid altering point mutations are normal variants. More likely such transcripts were generated from a mutated ER allele present in some breast tumours [30, 31] or are germline mutations [33, 34].

EVIDENCE THAT SOME VARIANT/MUTANT ER MRNAS ARE STABLY TRANSLATED *IN VIVO*

Recently, concern has been expressed with respect to the significance of variant ER-like mRNAs since little data had been available to support the existence of stable translation of the predicted ER-like proteins *in vivo*. This was despite the relative ease with which many of the respective proteins could be expressed at high levels from expression constructs transfected into mammalian, yeast and bacterial host cells. Interestingly, in some cases ER variant expression under these *ex vivo* conditions has identified a putative function of the resulting variant protein [12, 14, 16] (see Section 4).

However, more recently new data have emerged from several groups which strongly support the stable translation of at least some variant ER-like mRNAs *in vivo* in both normal and neoplastic human tissues as well as cell lines in culture.

An ER-like protein consistent with that predicted to be encoded by the exon 5 deleted ER transcript has been found to be expressed naturally in some BT 20 human breast cancer cell lines [35]. As well, an immunoreactive 80 kDa ER-like protein was identified in an MCF-7 subclone [36]. This protein corresponds to the predicted protein encoded by an ER-like transcript containing an exon 6 and 7 duplication cloned from the same cell line [30]. These data demonstrate the ability of some ER-like transcripts to be naturally translated into stable proteins, which can be detected by current methods and suggest the likelihood of other ER-like transcripts being stably translated *in vivo* under natural conditions.

More recently still, a group of human breast tumours were analyzed immunohistochemically [37] for ER expression using antibodies which recognize either an N-terminally localized epitope in the wild-type ER protein, or a C-terminally localized epitope in the wild-type ER protein. It was found that the antibody recognizing the C-terminally localized epitope correlated better with the ligand binding assays performed on adjacent tissues than did the antibody

recognizing the N-terminally localized epitope. As well, although in many tumours the immunohistochemical results using each antibody showed good concordance, in some tumours the results were discordant, with the signal tending to be higher with the N-terminal antibody [38]. Since many of the proteins predicted from variant ER mRNAs would be truncated at the C-terminus and not contain the epitope recognized by the C-terminal antibody, one interpretation of these data would be that truncated variant ER proteins are more highly expressed in the discordant group of tumours. This hypothesis was then tested by investigating the pattern and relative expression of variant ER mRNAs in the discordant and concordant groups of breast tumours. Several ER variant mRNAs which encode putative short ER-like proteins that would be recognized only by an N-terminal targeted antibody were preferentially and more highly expressed in the discordant breast tumour group. These ER variants were: the clone 4 truncated ER mRNA, the exon 2, 3 plus 7 deleted ER mRNA, the exon 2, 3 plus 4 deleted ER mRNA and the variant deleted within exon 3 to within exon 7 [38]. While this indirect approach does not specifically identify ER variant proteins, the data suggest that the ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER measured by immunodetection assays using N- or C-terminal antibodies.

Most recently, coexpression of an exon 4 deleted ER mRNA with the wild-type ER mRNA was also demonstrated in both normal and neoplastic ovarian tissue [39]. Most importantly, Western blotting analysis revealed the presence of both the wild-type 65 kDa ER as well as a 53 kDa immunoreactive protein. This is the predicted molecular mass of a protein encoded by an exon 4 deleted ER mRNA. While the 65 kDa protein was detected with ER antibodies recognizing several different epitopes along the length of the wild-type molecule, the 53 kDa protein was only recognized with antibodies recognizing epitopes outside of residues encoded by exon 4. An antibody which recognized residues within exon 4 failed to detect the 53 kDa protein [39].

The data described above together with other data present in the literature [16,40-44] strongly support the ability of ER variant mRNAs to be stably translated *in vivo*, supporting the speculation that ER variants have a functional role(s) possibly in ER signal transduction.

WHAT MIGHT BE THE FUNCTIONAL SIGNIFICANCE OF VARIANT/MUTANT ER EXPRESSION?

Several approaches have been used to investigate the possible significance of expression of ER variant/

mutant mRNA and the proteins they are likely to encode.

Recombinant expression vectors for some of the variant or mutant ERs transfected into mammalian and yeast host cells

This approach has identified putative functions for some of the resulting variant proteins [12, 14, 16]. For example, in HeLa [12] and breast cancer cells [45] but not yeast cells [46] the exon 3 deleted ER demonstrated dominant negative activity when coexpressed with the wild-type ER. The exon 4 deleted ER demonstrated dominant negative activity when coexpressed with the wild-type ER in COS-1 cells [39] but not rat vascular smooth muscle cells [47], embryo carcinoma P19EC cells nor human choriocarcinoma JEG3 cells [48]. In yeast cells [16] but not HeLa cells [12], an exon 7 deleted ER [16] variant acted as a dominant negative regulator of the wild-type ER [12]. Although no systematic, comparative studies have been reported to date, the data above suggest that cell type and possibly promoter specific activities of variant ER are likely to occur. Other ER deletion variants that have been found to inhibit wild-type ER activity are an exon 2 deleted ER in HeLa cells [12] and the exon 4 + 5 but not the exon 3 + 4 deleted ER in vascular smooth muscle cells [48].

None of the deleted ER variants described above have demonstrated any transcriptional activity irrespective of the presence or absence of estradiol. In contrast an exon 5 deleted ER demonstrated variable strengths of ligand independent transcriptional activity in yeast cells [14], chicken embryo fibroblasts [49] and MCF-7 human breast cancer cells [49]. A mutant ER mRNA isolated from T-47D_{co} cells contained a TT insertion such that the resulting protein is truncated after amino acid 250, immediately following the second zinc finger of the DNA binding domain [50]. Transient transfection of the appropriate expression vector into COS-1 and HeLa cells demonstrated low levels of constitutive transactivation activity as well as the ability to slightly inhibit the wild type ER activity. Also gel mobility shift assays demonstrated a low but detectable ability to bind to DNA [50]. The ER proteins encoded by inserted and exon duplicated ER mRNAs [29] have reduced or no transcriptional activity in COS-1 cells (Douglas and Murphy, unpublished data).

The 3', C-terminally truncated ER variants such as clone 4 [28] do not show transcriptional or inhibitory activity in COS-1 cells [28], while 5', N-terminally truncated ER-like molecules identified in rat pituitaries may enhance the transcriptional activity of the wild-type ER [51].

Alteration of individual variant ER expression by stable transfection

A study done by Fuqua and Wolf [52] showed that over-expression of the exon 5 deleted ER protein by

stable transfection of MCF-7 human breast cancer cells resulted in increased progesterone receptor levels in the absence of estrogen, as well as estrogen independent growth and tamoxifen resistance. In contrast, a similar study done by Rea and Parker [49] showed no effect on endogenous estrogen responsive genes such as pS2 and progesterone receptor, neither did these cells demonstrate estrogen independent or anti-estrogen resistant growth responses. The reasons for the different results between the two groups are unclear, although differences in the original parent MCF-7 cells was suggested, in turn suggesting that changes in addition to altered exon 5 deleted ER expression are required for hormonal progression in human breast cancer cells. This is not unreasonable since several mechanisms either alone or in combination may be responsible for such progression [54]. For example, it is possible that the alteration of growth factors or their cognate receptors, some of which have been shown to result in ligand independent activation of the wild-type ER through the N-terminal AF-1 domain [5, 53], may also be required in conjunction with altered expression of ER variants.

A naturally occurring ER mutation (aspartate 351 → tyrosine) originally isolated from a tamoxifen-stimulated MCF 7 breast tumor growing in athymic mice, demonstrated an increased estrogenicity of 4-hydroxytamoxifen [55] after stable transfection of an expression vector into MDA MB 231 breast cancer cells. Such activity would be consistent with a functional involvement of the mutated receptor in tamoxifen stimulated growth in this tumor. However, not all tamoxifen stimulated MCF 7 breast tumors in athymic mice contain mutated ER [55].

Determination of expression of individual ER variants in normal and disease tissues

Several ER variants have now been identified in normal tissues including human breast [17–19, 56], human uterus [14, 24, 25] and human ovary [39], as well as rat brain [27, 57]. Such data imply that ER variants have a normal physiological role and altered expression of these variants may be one mechanism contributing to disease processes such as tumorigenesis. In particular it has now been demonstrated that the relative expression of the clone 4 truncated ER mRNA is increased in breast tumors compared to normal breast tissue [56]. Furthermore, increased expression of the exon 5 deleted ER mRNA but not the exon 7 deleted ER mRNA was also observed in breast tumors compared to normal breast tissue [17]. In contrast, it has been suggested that decreased expression of the exon 3 deleted ER mRNA occurs in breast tumors compared to normal breast tissue [45].

Interestingly, the level of an exon 5 deleted ER transcript was relatively higher in hepatocellular carcinoma tissue compared to non-tumorous liver tissue from male patients with cirrhosis [58] and lack of

responsiveness to endocrine therapy was correlated with increased ER variant mRNA [59]. As well preliminary data suggest that differential expression of ER variant and wild-type ER in peripheral blood mononuclear cells may exist between normal females and female lupus patients [60].

Correlation of ER variant expression with known prognostic variables in breast cancer

The expression of several ER variant mRNAs has been correlated with prognostic variables, which would be consistent with a role for such variants either functionally or as markers in breast cancer. The relative level of expression of the clone 4 truncated ER mRNA was found significantly elevated in breast tumors with characteristics of poor prognosis and endocrine resistance vs those with characteristics of good prognosis and endocrine sensitivity [61]. Interestingly, the relative level of an exon 5 deleted to the wild-type ER mRNA was increased in hepatocellular carcinomas from patients who later responded poorly to an endocrine therapy [59]. The exon 5 deleted ER transcript was first identified in a tumor that was ER - /PgR +, and was found at significantly higher levels in those human breast tumors which were ER - /PgR+ or ER - /pS2+ [62], findings which are consistent with the speculation concerning the ligand independent activity of a protein encoded by an exon 5 deleted ER mRNA [14]. However, the relative levels of expression of this variant to other ER variant mRNAs in a range of human breast tumors appears to be quite low and not detected in some cases [21, 63].

Fuqua *et al.*, originally isolated the exon 7 deleted ER variant mRNA from an ER + /PgR- breast tumor which contained an ER-like protein able to bind to DNA containing an estrogen responsive DNA element (ERE) as determined by gel mobility shift analysis, but which interacted differentially with ER antibodies suggestive of an ER-like protein with a C-terminal truncation [16]. The demonstration that the exon 7 deleted ER protein could inhibit wild-type ER activity [16] was consistent with the idea that over expression of an exon 7 deleted ER protein could contribute functionally to the ER + /PR- breast tumor phenotype. This hypothesis was further supported by the observation that exon 7 deleted mRNA levels were significantly elevated in a group of human breast tumors that were ER + /PgR - /pS2- compared to those which were ER + /PgR +. However, it should be remembered that the ability of an exon 7 deleted ER variant to inhibit wild-type activity has only been found in yeast cells but it was inactive in human HeLa cells [12].

More recently, a correlation was found between the relatively increased expression of the exon 4 deleted ER mRNA with high PR expression and low grade, suggesting its association with some good prognostic

features in human breast tumors [21]. In this same study, the detection of the exon 2 + 3 + 4 deleted transcript was significantly correlated with high grade tumours [21].

Correlation of variant or mutant ER expression with altered estrogen and antiestrogen responsiveness in vivo and in cell lines in culture

Klotz *et al.*, [64] identified a correlation between increased expression of the exon 5 deleted ER transcript relative to the wild-type ER and reduced responsiveness to estrogen in MCF-7 stocks obtained from various laboratories in North America. In addition, an exon 2 deleted ER mRNA was found to be over-expressed in a tamoxifen resistant MCF-7 cell line compared to the parent MCF-7 cells, although other ER variant transcripts were also differentially expressed in these two cell lines [20]. An 80 kDa immunoreactive ER-like protein encoded by an ER mRNA containing a duplication of exon 6 + 7 was recently reported in an MCF-7 subclone, which was estrogen independent with respect to growth [30, 36]. More recently, the relative expression of an ER mRNA deleted in exons 3 + 4 was found markedly increased in human breast cancer cells which have become estrogen independent (Coutts *et al.*, unpublished data). Although such data support a role for altered ER variant expression in hormone independence, the mechanism(s) by which this is achieved remain unclear as yet.

However, no correlation of ER mutations and/or variants with clinical de novo and/or acquired tamoxifen resistance in breast cancer patients *in vivo* has been obtained [23, 62]. Interpreting such findings to mean that ER variants do not have a role in tamoxifen resistance should be tempered by the likelihood that tamoxifen resistance is multifactorial [54] and the clear demonstration that multiple ER variants can occur together in any one tumor sample [19, 21].

Interestingly, Fuqua *et al.*, [65] have identified a mutated ER, which is super-sensitive to estrogen, at high frequency in atypical hyperplastic breast lesions. This observation would be consistent with the hypothesis that ER signalling is progressively increased in preneoplastic breast lesions and may have a role in the evolution of breast cancer [11].

Other evidence supporting the involvement of ER mutations and variants in disease processes is the identification of a germline mutation in the human ER in a young adult male presenting with osteoporosis, unfused epiphyses, continued linear growth in adulthood, and estrogen resistance. This study importantly demonstrated that disruption of the ER need not be lethal in humans and identified the importance of estrogen in bone maturation and mineralization in men as well as women [33]. In a very small number of cases a mutation or polymorphism in the ER gene might be linked to late onset hereditary breast

cancer [34]. As well, the first point mutation that was identified in the human ER was an G to C mutation [66–68] at nucleotide 261 (using the numbering present in Ref. [69]). Although this is a silent polymorphism, interestingly this so-called B region variant allele (B') of the ER has been correlated with decreased levels of estrogen binding in human breast cancers [70], increased history of spontaneous abortion in women with ER positive breast cancer [71], increased height in women [72] and possibly increased prevalence of hypertension [73].

Tissue specific expression of ER variants

Both tissue and gender specific expression of ER variant mRNAs have been documented. The 5'-truncated ER variants are detected only in the female rat pituitary [27]. This type of variant is not present in the female rat uterus, hypothalamus, ovary or liver, neither is it present in male rat pituitary. However, the 5'-truncated ER variants are induced in male pituitary if the animal is treated with estrogen [27, 74]. A novel splice variant of the ER mRNA consisting of a deletion from within exon 4 to within exon 7, was detected in human endometrial tissue and breast cancers but not in normal liver tissues [24]. These data suggest that the pattern of ER variant expression can differ amongst normal target tissues and may be altered by hormones. Such data would be consistent with the hypothesis that the physiological role for ER variants may underly tissue specific differences in estrogen and possibly antiestrogen action. This speculation becomes increasingly attractive given recent studies reporting the characterization of novel estrogen responsive DNA sequences, which remain quite distinct in structure–function activity and presumably mechanism from that determined using classical ERE sequences from the vitellogenin promoter [75–78].

SUMMARY AND CONCLUSIONS

The above review shows that there is a large and increasing body of experimental and clinical data supporting the existence of variant and mutant ER proteins. Therefore, future examination of ER signal transduction and/or measurement of ER protein must take into account variant and/or mutant ER expression. The functions of variant ER proteins, either physiological or pathological, remain unclear, although a role(s) for some ER variants in breast tumorigenesis and breast cancer progression would be consistent with the data so far accumulated. However, future speculation concerning these issues must take into account co-expression of the wild-type ER with multiple ER variants in any one tissue sample, as well as the relative expression of each with respect to others. Indeed, this parameter seems to be altered in different groups of breast tumours, as discussed above. The possibility that the pattern of ER variant

expression can differ amongst different normal estrogen target tissues leads to the exciting speculation that this may be one of the mechanisms underlying tissue specific differences of ER signal transduction. Although this remains to be systematically studied, it is clear from the discussion above that analyses of putative function of individual ER variants must consider the cellular and promoter context together with the presence or absence of wild-type ER and other ER variants. The future investigation of ER variant function promises to be exciting, especially in the light of recent studies where novel estrogen responsive DNA sequences have been identified, which remain quite distinct in structure–function activity and presumably mechanism from that classically determined using ERE sequences from the vitellogenin promoter [75–78].

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APPENDIX 20

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Estrogen receptor mRNA variants influence the determination
of ER status in human breast breast cancer.

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Influence of Estrogen Receptor mRNA Variants on the Determination of ER status in Human Breast Cancer

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Keywords: Estrogen variant mRNAs, Estrogen receptor status, immunohistochemistry, breast cancer

Abstract

Background: Estrogen receptor alpha (ER) status in breast cancer is an important predictive factor for clinical response to endocrine therapy. We have recently shown that discrepancies in ER status determined by immunohistochemical assay (ER-IHA) can occur between different ER antibodies and that this may be attributable to the expression of truncated ER variant proteins. In this study we have examined the direct effect of ER variant expression on ER-IHA and whether discrepancies in ER-IHA status are reproducible with different antibodies.

Methods: ER negative Cos-1 cells were transiently transfected with expression vectors containing wild type ER (wt-ER) and/or the truncated ER-Clone-4 variant. Expression of ER-like proteins was determined by Western blot and ER-IHA using ER specific monoclonal antibodies against different epitopes of the ER protein and quantitated by both densitometry and H-score analysis. Statistical comparisons were assessed by the student t-test. Paraffin sections from 10 tumors were also studied by ER-IHA.

Results: ER-IHA with N- and C-terminal ER antibodies on Cos-1 cells expressing wt-ER alone demonstrated no difference in signals by Western blot ($P>0.1$). However co-expression of wt-ER and the truncated ER-clone-4 variant, resulted in discordant IHA results with relatively higher ER-IHA scores from N-terminal antibodies ($P<0.03$). Furthermore, re-examination of breast tumors previously studied by ER-IHA showed persistent differences in 8/10 cases with a different pair of ER antibodies.

Conclusions: We conclude that truncated ER variant proteins can interfere with IHA determination of ER status and that this may account for some of the inconsistencies between ER status and response to endocrine therapy.

Introduction

The measurement of estrogen receptor alpha (ER) status in breast tumors is widely used as a clinical index of potential therapeutic response to endocrine therapy. However, while up to 2/3 of breast tumors are ER positive, only 2/3 of this subset of patients will respond well to endocrine therapy (1). Several factors have been considered in the past to account for this discrepancy, including tissue related factors such as sampling, cellularity and heterogeneity and biological factors such as functional status of the ER protein detected and the integrity of the downstream components of the ER signalling pathway.

More recently other potentially important biological factors that have emerged are the complexity of ER alpha gene expression as well as the recent discovery of the closely related ER beta gene that is also expressed in breast cancer tissues (2). ER alpha gene expression is now known to be often associated with a range of ER variant mRNAs in breast tumors (3). These ER mRNA variants include exon-deleted, exon-duplicated or truncated ER mRNA transcripts that may encode a variety of incomplete ER-like proteins (3,4). Individual ER variant proteins have been demonstrated (5,6) but in most instances may well be expressed at low levels in most tumors compared to wt-ER (4). Nevertheless ER variant expression may be important to consider with the adoption of the IHA as an alternative to the classical ligand binding dextran coated charcoal (DCC) assay to determine ER status (7,8,9). The IHA affords the opportunity to determine ER status in paraffin tumor sections and so allows the parallel assessment of tissue factors. However this has also meant that ER is now defined on the basis of structural epitopes as opposed to functional ligand binding. Expression of most ER variants would not be detected by the DCC assay because in many cases the predicted variant proteins have loss or disruption of the C-terminus and ligand binding domain (E/F region) of the protein (10). However, the total accumulation of multiple ER-like proteins might well be

expected to interfere with IHA determination of ER status, depending on the target specificity of the antibody employed. Thus, although in practice a good overall correlation between IHA and DCC exists, discrepant results occur in a proportion of tumors (8,9,11,12,13). These discrepancies are not only between IHA and DCC assays (8,9), and also between IHA performed with different antibodies on the same tumors (11,12,13). While tissue related factors can be invoked to account for some of the former discrepancies, differences between comparable IHAs scored on the same areas within serial sections (11) are more difficult to explain.

In order to understand such discrepancies we have recently examined ER mRNA variant expression and shown that those variants that specifically encode putative truncated ER-like proteins, are preferentially expressed in these 'IHA-inconsistent' cases (14). This suggests that ER variant proteins encoded by ER variant mRNAs may contribute to discrepancies in ER status determined by IHA using different antibodies. In this study we have now compared the signal intensities of different ER targeted antibodies and used these to examine experimentally the direct effect of truncated ER variant expression on the determination of wild type ER (wt-ER) status by IHA.

Materials and Methods

ER expression vectors and transfection assays

Wt-ER (HEGO, kindly provided by Dr P. Chambon) was cloned into the vector pSG5 and expression was driven by an SV40 promoter. Truncated ER-clone-4 was cloned into the pcDNA3.1 vector (Invitrogen) and expression was driven by a CMV promoter. ER-negative Cos-1 cells were grown in DMEM supplemented with 5% (v/v) fetal bovine serum. The cells were transiently transfected with 5µg of either pHEGO or pER-clone-4 expression plasmid, or both in varying

proportions (as described in Fig 2). After 48 hours, transfected cells were harvested for immunoblotting or fixed for immunohistochemical assay. All transfections were done in 60-mm dishes (for Western blot samples) or in chamber slides (for immunohistochemistry samples) in parallel and using the Superfect transfection system (Qiagen, CA) as described by the manufacturer. Plasmid pCH110 (encoding β -galactosidase protein, Pharmacia) was co-transfected and galactosidase activity was determined by standard methods to control for and confirm transfection efficiency (25-35% cells positive).

Western Blot Analysis

Whole cell extracts were prepared from cells transfected with wt-ER, ER-clone-4 or both plasmids. Cells were washed with chilled phosphate-buffered saline (PBS), scraped, collected in PBS, centrifuged at 1000g for 5 minutes at 4°C and cell pellets were resuspended in 200 μ l of 50 mM Tris-HCl, 20 mM EDTA, 5% Sodium dodecyl sulphate(SDS), 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM β -glycerophosphate and 1mM aprotinin. Protein concentration was determined by the Bio-Rad (Hercules, CA) protein assay kit as described by the manufacturer. Twenty-five micrograms of protein were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Western blot analysis to detect ER present in different transfected cells was carried out with ER-specific mouse monoclonal antibodies 1D5 (DAKO, Canada) or AER314 raised against N-terminal epitopes and AER311 or AER320 (Neomarkers, CA), raised against C-terminal epitopes (at 1/1000 dilution for all antibodies) of the wild type protein (Fig 1D). The second antibody used was a horse-radish peroxidase (HRP)-conjugated goat anti-mouse antibody (Hyclone Laboratories, Logan, UT, USA). Visualization was accomplished using the Supersignal detection system (Pierce, USA.) according to the manufacturer's instructions. Densitometry on Western blot signals was performed using a video-computer image analysis system (M4, Imaging

Research, St Catherines, Ontario). All Western blot experiments were performed at least in triplicate on independent cell transfections. Statistical comparisons between antibody signals were assessed by the student t-test.

Immunohistochemistry

In parallel experiments, Cos-1 cells were grown in chamber slides (Nalge Nunc, Intl., IL, USA). Transfections were performed as above. Cells were fixed in 2% paraformaldehyde-PBS for 30 minutes and washed twice with PBS for 2 minutes. Then IHA was performed as described previously (11). Briefly, the slides were incubated in 10% goat serum in PBS for 20 minutes to block non-specific binding. Primary antibody 1D5 or AER311 (at 1/50 dilution for both) was added and incubation carried out overnight at 4°C, followed by two washes in PBS for 5 minutes. The second antibody (biotinylated goat anti-mouse IgG, Vector Labs, CA) was used at 1:200 dilution in PBS for 45 minutes at room temperature. After a rinse in PBS, the slides were incubated in AB Complex (Elite kit, Vector Labs) at 1:100 dilution for 45 minutes. The label was developed using diaminobenzidine/hydrogen peroxide and slides were then lightly counterstained with methyl green, dehydrated, cleared and mounted.

Human breast tumor specimens (10 cases) were obtained from the NCIC-Manitoba Breast Tumor Bank. Formalin fixed and paraffin embedded tissue blocks were sectioned to provide serial sections from tumors and examined by IHA using the same protocol except that a different pair of ER antibodies (AER314 and AER320, Neomarkers, CA) were used.

Semi-quantitative H-scoring for all IHA experiments was done as previously described (11). Brown immunoreactivity of cell nuclei was taken as positive and the proportion of negative cells (P_0) and those staining at low (P_1), moderate (P_2), or high (P_3) levels of intensity scored. The score for each section ($H\text{-score} = [(0 \times P_0) + (1 \times P_1) + (2 \times P_2) + (3 \times P_3)] \times 100$) was calculated from the

mean of x 5 representative high power fields (Leica DMRB, x40 objective). For transfection experiments where non-specific background was uniformly higher than in tumor sections, only P_2 and P_3 values were entered into the final H-score. Initial IHA experiments were also analyzed with the video-densitometry system as above, to compare with and validate the H-scoring quantification. All IHA slides were coded and assessment was done without knowledge of the antibody, transfection conditions, or tumor identity. As described previously (11), tumors which exhibited an H-score difference of >50 between IHA assays performed with different ER antibodies on serial sections were classified as 'IHA-inconsistent'.

Results.

Comparison of ER antibodies.

A panel of ER antibodies directed to different epitopes was tested by IHA and Western blot assay in parallel, applied to an ER negative Cos-1 cell line transfected with wt-ER. All antibodies detected a 65kDa protein on Western blot (Fig.1A) and comparison using a video-densitometry system showed that there was no significant difference between the signals obtained with 1D5 and AER311 antibodies (optical density units mean^{SD} for 1D5 = 0.79^{0.22} vs mean^{SD} AER311 = 0.78^{0.19}; n = 4, P>0.1). IHA was performed in parallel on transfected cells using these antibodies at the same relative concentrations. These were also the concentrations that we had previously used to study breast tumors. Initially IHA was assessed by both video-densitometry and semi-quantitative H-score (applied independently and without knowledge of the antibody) and it was found that these provided comparable results (n = 10, r = 0.96, p = 0.004, data not shown). Subsequently all IHA signals were quantitated by H-score. Positive immunoreactivity in approximately 30% of nuclei was seen with IHA-1D5 and IHA-AER311 with no significant difference in ER levels (H-score 1D5 mean^{SD} = 63⁸; AER311 mean^{SD} = 60¹⁷, n = 7, P>0.1, Fig.2D).

Effect of modulation of ER variant expression on IHA.

The ER-clone-4 variant was used for these experiments as this variant is predicted to encode a C-terminally truncated ER-like protein and has previously been shown to be frequently expressed in breast tumors (14,15,16). Following transfection of ER-clone-4 alone, a single 24 kDa protein was detected by Western blot analysis (Fig.1B) and positive nuclear staining was only seen by IHA using N-terminal antibodies (1D5 and AER314). However, C-terminal antibodies (AER311 and AER320) failed to detect it on Western blot (Fig 1B&C) or IHA (Fig.2D). IHA was then performed using the same protocol as used in our previous study of breast tumors applied to cells following co-transfection with ER-clone-4 variant and wt-ER to obtain different proportions of ER variant expression relative to wt-ER. We observed that while both antibodies recognized wt-ER on Western blot (Fig.1C) and gave similar H-score values with wt-ER alone, a significant and increasing discordance in IHA H-score occurred between 1D5 and AER311 as the relative proportion of ER-clone-4 variant increased ($P < 0.03$, t-test, Fig 2D).

ER-IHA on breast tumors.

We then investigated the reproducibility of our original observation in breast tumors. A different pair of antibodies was selected for repeat IHA assay (AER314 and AER320 targeting N- and C-terminal ER epitopes respectively) as these were found to give similar results in Cos-1 cells following wt-ER transfection and Western blot analysis (AER314 mean^{SD} = $0.67^{0.28}$; vs AER320 mean^{SD} = $0.65^{0.22}$; $n=4$, $P > 0.1$) and IHA (data not shown). The original tissue blocks were available on 10 cases that were all ER/PR positive by DCC assay. All 5 tumors that had previously been classified as 'IHA-inconsistent' by our previous definition (H-score difference > 50) showed lower H-scores by IHA-AER320 as compared to IHA with the matching AER314

antibody. In 3/5 this was sufficient to remain classified as 'IHA-inconsistent' (Table 1). In contrast, amongst an equal number of cases previously classified as 'IHA-consistent', 3/5 showed equivalent or higher H-scores with AER320. In 1/5 of the latter cases the IHA was inconsistent.

Discussion.

Multiple ER alpha mRNA variants are expressed in normal breast tissue and in breast tumors (3). However, in considering the role of ER variants in breast cancer, it has been argued that expression of ER mRNA variants may not be important on the basis that expression may not change during tumorigenesis and that the evidence to date for expression of specific variant proteins that might play a role in hormonal progression is limited (17,18). Nevertheless, studies founded on histologically characterised tissue sections have clearly shown that certain mRNA variants are differentially expressed between normal and neoplastic tissues and also between tumors (19,20). Differential expression has also been found in association with contrasting responses to estrogen and resistance to tamoxifen in cell lines (21,22,23,24) and parameters of hormone response and prognosis in-vivo (16,25). Overexpression of an ER mRNA variant deleted in exon 5 (D5-ER) has also been shown to occur in certain tamoxifen resistant tumors (26) and has been used successfully to predict response to hormonal therapy of hepatocellular carcinoma (27). It is also conceivable that ER variants might exert indirect functional effects through competition with wt-ER for ER binding proteins (28) or with proteins involved in interactions with antiestrogens (29).

Although expression of specific variant proteins has not yet been proven in breast tumors, partly because it has been difficult to develop antibodies that will distinguish variants, expression of ER-like variant proteins expressed recombinantly can be demonstrated in-vitro and in breast cell lines (21,22). In some cases these variant proteins have been shown to possess either hormone independent and constitutive activity or to exert a dominant negative influence on estrogen regulated target genes

in ex-vitro models. At the same time our own data suggest that previous attempts to demonstrate these proteins in-vivo may have been hampered by the fact that, although total expression of ER variants of all forms may be significant, many individual variants, such as D5-ER may be expressed at only low levels in breast tissues (4). Furthermore, our recent observation that inconsistent immunostaining with ER antibodies correlates with the total overall expression of mRNA variants encoding out-of-frame proteins (i.e. predicted to encode C-terminally truncated ER-like proteins) also supports the view that ER-like variant proteins are present in-vivo (11,14). In the latter study we examined ER expression in breast tumors by DCC and IHA using both 'N-terminal' (1D5) and 'C-terminal' (AER-311) targeting ER alpha antibodies. The IHA provided similar results to the DCC assay in terms of ER status. However the ER-IHA results from almost 25% of tumors were discordant (H-score difference of > 50) between these different antibodies, even when scored on the same areas within serial sections (11). Further analysis of these 'IHA-inconsistent' cases by RT-PCR assays (4,20) showed that those ER mRNA variants that encode putative truncated ER-like proteins, were preferentially expressed in 'IHA-inconsistent' cases (14).

However, although the antibodies we used provided similar signal intensities by IHA on strongly ER positive tumors, and in many tumors the IHA signals were concordant (11), it remained possible that the discordant IHA signals might be explained by different antibody affinities. Furthermore, the principle that ER variants may interfere with IHA results had not previously been tested experimentally. Our results here show that the 1D5 and AER-311 antibodies can provide similar signal intensities of wt-ER by both Western blot and IHA when used at the same relative concentrations as in our previous study (11). But the relative IHA signal intensity obtained with these antibodies changes and becomes inconsistent with increasing expression of a truncated ER variant protein alongside the wt-ER. IHA consistency or inconsistency is also reproducible when tumor blocks that have previously been studied are re-

examined using a different pair of matched ER monoclonal antibodies targeting similar N and C terminal epitopes. In one case the paired IHA results were quite different between these studies. However the precise epitopes recognized by all the ER antibodies used is unknown. Also the presence of additional truncated variants in this particular case, similar to ER-clone-4, that would remain undetected by the RT-PCR assays we have previously used, cannot be ruled out (15,16).

In summary, we have shown that ER status determined by IHA can be directly influenced by expression of a C-terminally truncated ER variant and that inconsistent IHA results in tumors are reproducible. We conclude that ER variant proteins can interfere with IHA assessment of ER status and that this may underlie some of the inconsistencies in determination of ER status in breast tumors. The relationship between ER variant expression, 'IHA-inconsistent' status and clinical response to endocrine therapy remains to be determined.

Figure Legends

Figure 1. Western blot analysis of estrogen receptor expression detected with different antibodies in Cos-1 cells following transient transfection with ER and/or ER-clone-4 variant. Twenty five μ g of whole cell extract protein prepared from transfected Cos-1 cells were loaded in each lane and separated by 0.1%SDS-10%PAGE. (A) Cos-1 cells transfected with wt-ER. (B) Cos-1 cells transfected with ER-clone-4 (C) Cos-1 cells transfected with both wt-ER and ER-clone-4. Monoclonal antibodies used to detect ER proteins were 1D5 or AER314, and AER311 or AER320, which target epitopes within the N-terminal and C-terminal of the ER protein, respectively (D).

Figure 2 Immunohistochemical detection of estrogen receptor-like protein in transfected Cos-1 cells. IHA-1D5 assay (A, B) and IHA-AER311 assay (C) were conducted on cells transfected with either ER-wt alone (A), or co-transfected with ER-wt and ER-clone-4 (ratio 1%:99%, B&C). The graph (D) summarizes the H-score values obtained by either IHA-1D5 or IHA-AER311 applied to Cos-1 cells transfected with ER-wt and ER-clone-4 in different proportions. Each bar represents the mean and standard deviation relative to 1D5 H-score applied to ER-wt transfected cells derived from 7 (ER-wt alone) or 3 (all other) independent transfection experiments.

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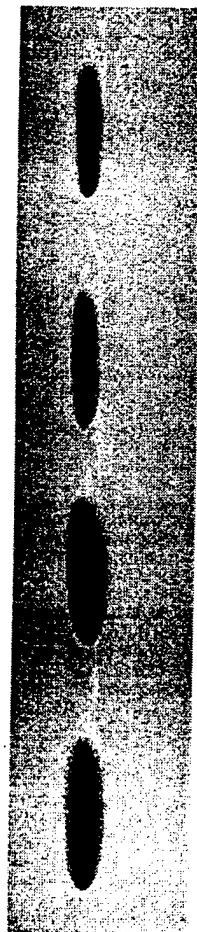
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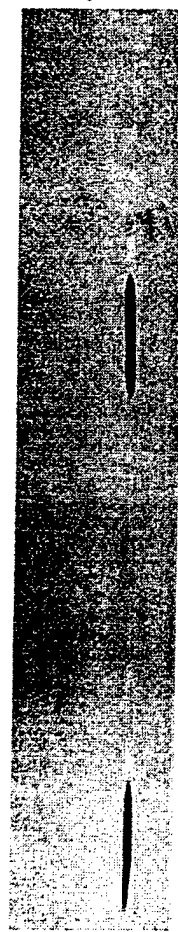
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1D5 311 314 320



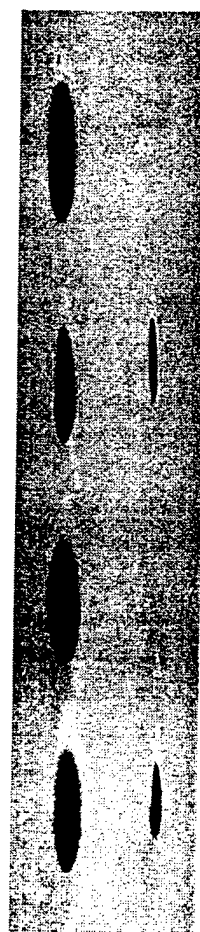
wt-ER

A



ER-Clone-4

B



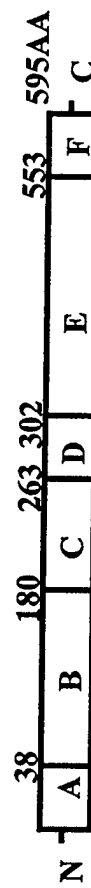
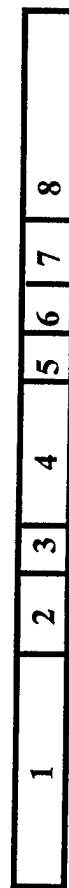
wt-ER

ER-Clone-4

C

wt-ER mRNA

D



wt-ER protein

1D5 — AER-311

AER-314 — AER-320

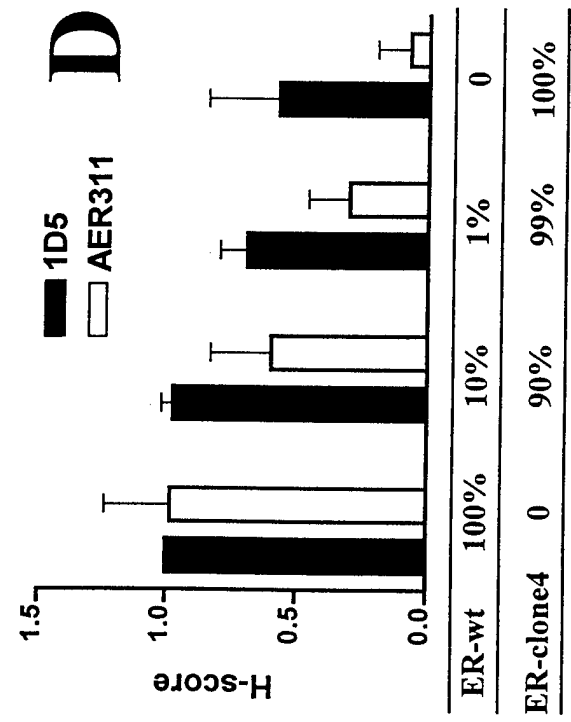
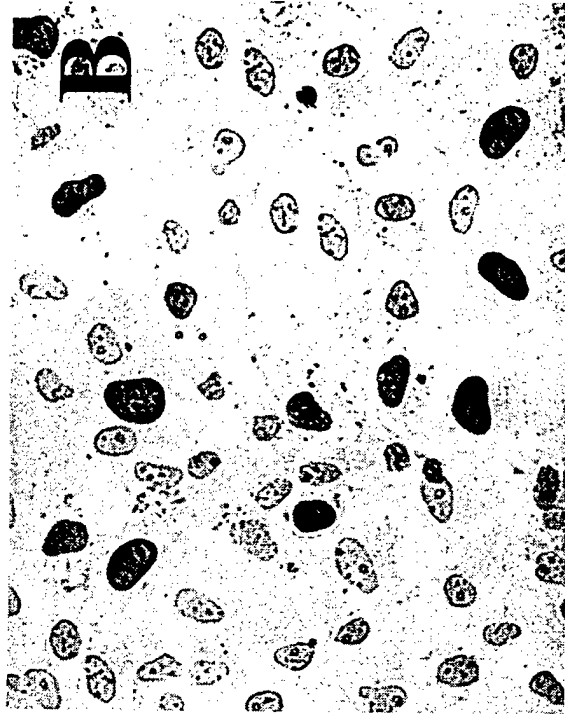
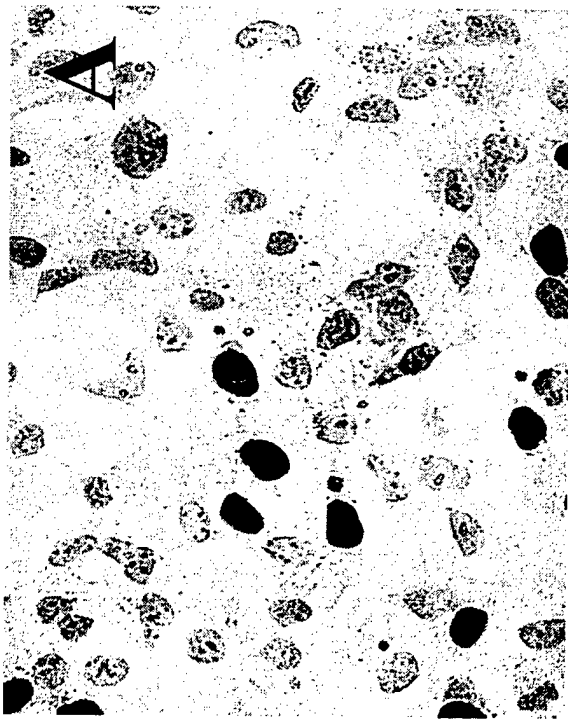


Table 1 H-Score determination by immunohistochemical assay with different ER antibodies

Tumor #	ER	PR	ID5	AER311	ID5↔311	AER314	AER320	314↔320
1	69	18	200	100	100	150	80	70
2	7	26	134	14	120	180	90	90
3	33	21	182	88	94	110	60	50
4	140	101	160	66	94	125	100	25
5	19	10	190	20	170	90	80	10
6	39	44	132	128	4	105	140	35
7	79	22	124	138	14	150	170	20
8	85	125	120	150	30	150	150	0
9	27	10	228	200	28	165	135	25
10	24	59	128	104	24	150	50	100

ER / PR: estrogen / progesterone receptor level determined by DCC assay (fmol/mg protein)

ID5, AER311, AER314, AER320: estrogen receptor level (H score values) determined with the corresponding antibody.

ID5↔311, 314↔320: Difference in H-score values with each antibody pair. Inconsistent H-Score between antibodies (difference >=50) shown in bold, consistent H-Score between antibodies (<50) shown in regular typeface.

APPENDIX 21

Huang A, **Leygue E**, Snell L, Murphy LC, and Watson PH

Expression of estrogen receptor variant mRNAs and determination of estrogen receptor status in human breast cancer.

Am J Pathol, 150: 1827-1833, 1997.

Expression of Estrogen Receptor Variant Messenger RNAs and Determination of Estrogen Receptor Status in Human Breast Cancer

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Estrogen receptor (ER) status of breast cancer can be assessed by immunohistochemical assay (IHA), although we have previously observed that ER-IHA levels can be inconsistent between amino-terminal and carboxyl-terminal-targeted antibodies. To address the hypothesis that this discrepancy is attributable to expression of ER variant mRNAs encoding truncated ER-like proteins, we have studied 39 IHA-consistent and 24 IHA-inconsistent breast tumors by reverse transcription polymerase chain reaction to examine the expression of multiple exon-deleted (D-ER) variant mRNAs and the truncated ER clone 4 variant mRNA. ER variants D7-ER, D4-ER, D3-4-ER, and D4-7-ER were detected at similar frequencies in both groups. However, ER variants D2-3/7-ER, D2-3-4-ER ($P < 0.05$), and D-3-7-ER ($P < 0.01$), which encode putative short ER-like proteins that might be recognized only by an amino-terminal-targeted antibody, were preferentially detected in inconsistent cases. ER clone 4 mRNA expression was also higher in inconsistent tumors ($P < 0.001$). Further analysis showed that, whereas overall prevalence of ER variant mRNAs was similar in both tumor groups, occurrence of the subset of variant mRNAs encoding putative truncated proteins was also higher in IHA-inconsistent tumors ($P < 0.05$). These data suggest that ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER-IHA levels determined using amino- or carboxyl-terminal-targeted antibodies. (Am J Pathol 1997, 150:1827-1833)

Estrogen receptor (ER) determination is an important parameter in the clinical management of breast cancer.^{1,2} Until recently, ER content was assessed principally by ligand-binding techniques such as dextran-coated charcoal (DCC) or sucrose gradient assays. Now, with the development of several antibodies able to recognize ER protein, immunohistochemical assay (IHA) has become an alternative approach to determine ER status of breast tumors and to predict endocrine response in breast cancer.^{3,4} The ER-IHA approach has significant advantages including the potential for parallel assessment of tumor cell content and heterogeneity of ER expression. However it differs from traditional methods in that ER activity is defined by structural rather than functional criteria.

ER-IHA in tissue sections has been successfully achieved by several different antibodies, including 1D5, H222, and AER311, which are able to recognize different epitopes within particular domains of the ER protein (Figure 1).³⁻⁵ However, we and others have previously observed that the ER-IHA results from some tumors are discordant between different antibodies that are able to recognize either the NH₂ or the COOH terminals, with a tendency to higher signals with NH₂-terminal-targeting antibodies.^{4,5} Although these differences might relate to different antibody affinities, another explanation lies in the existence of ER variants. Beside the wild-type ER mRNA transcript, several ER variant mRNAs have been described in both normal and cancer tis-

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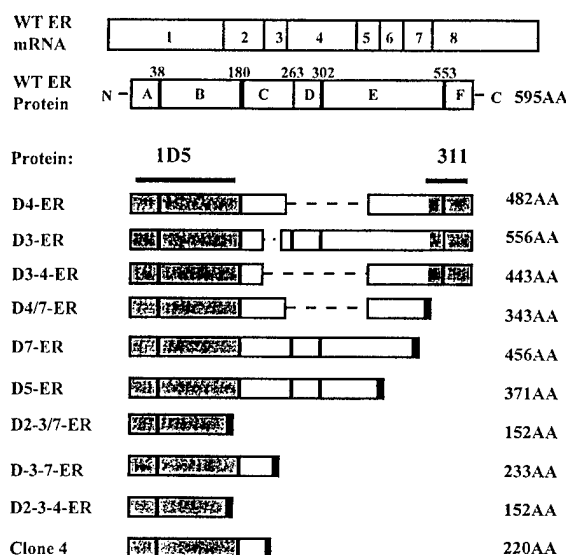


Figure 1. Schematic presentation of WT-ER protein and the predicted proteins encoded by ER variant mRNAs. ER protein contains A to F functional domains. Region A/B of the receptor is implicated in trans-activating function (TAF1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (TAF2). WT-ER reading frame is conserved in ER variant mRNAs deleted in exon 4, in exon 3, and in both exons 3 and 4. Encoded proteins from D4-ER, D3-ER, and D3-4-ER, respectively, are similar to WT-ER (open box) but miss some internal amino acids (- - -). Simple deletion of exon 7 or exon 5 and multiple deletion of exon 4 and exon 7; exons 2, 3, and 7; exons 3 and 7; and exons 2, 3, and 4 introduce a shift in the ER-WT reading frame. The resulting proteins, D7-ER, D5-ER, D4/7-ER, D2-3/7-ER, D-3-7-ER, and D2-3-4-ER, respectively, are therefore similar to WT-ER (open box) but are truncated of the C-terminal WT region (black box, indicating amino acids different from WT-ER). Clone 4 protein is encoded by an ER variant mRNA containing WT-ER exon 1 and exon 2 juxtaposed with line-1-related sequences. Clone 4 protein is similar to WT-ER (open box) but is missing the C terminal. The gray areas represent regions of the protein that are theoretically recognized by 1D5 or AER311 antibodies.

sues.⁶⁻¹⁴ Most of these variants are suspected to result from alternative splicing of WT-ER mRNA and consist of exon-deleted and truncated variants.^{6,8} Figure 1 shows some of the putative proteins encoded by these variants and illustrates that, whereas some of these altered proteins may still possess both NH₂- and COOH-terminal epitopes of the wild-type (WT) protein, others will be truncated and lack the COOH terminal as a result of an exon deletion that introduces a shift in the reading frame. In addition to exon-deleted ER mRNA variants, several truncated variants have been described, among which the ER clone 4 variant is highly prevalent in breast tumors.⁸ The sequence of this variant mRNA corresponds to WT-ER exon 1 and 2 juxtaposed to line-1-related sequences, and *in vitro* analysis shows that it encodes a putative ER-like protein missing the carboxyl-terminal extremity.

To address the hypothesis that discrepancies observed by IHA using 1D5 and AER311 antibodies in breast tumors could result from particular ER variant

expression, we investigated 39 IHA-consistent and 24 IHA-inconsistent breast tumors for the most prevalent exon-deleted ER variant mRNAs and in parallel for the level of ER clone 4 truncated variant mRNA expression by two reverse transcriptase polymerase chain reaction assays that we have recently developed to assess multiple ER variants in breast cancer tissues.^{15,16}

Materials and Methods

Human Breast Tissues and ER Status Determination

The study was carried out on 63 cases of invasive ductal and invasive lobular breast carcinomas obtained from the NCIC-Manitoba Breast Tumor Bank.¹⁷ These cases correspond to the ER-positive subset of a series of 97 tumors previously studied by IHA.⁵ In all cases, the specimens had been rapidly frozen at -70°C as soon as possible after surgical removal. Subsequently, a portion of the frozen tissue from each case was processed routinely to create formalin-fixed, paraffin-embedded tissue blocks that were matched and orientated relative to a corresponding frozen tissue block. Paraffin sections were previously analyzed by IHA using 1D5 (Dako, Dimension Labs, Mississauga, Canada) and AER311 (Neomarkers, Lab. Vision Corp., Fremont, CA) ER monoclonal antibodies.⁵ In each case, immunohistochemical staining was assessed, without knowledge of the ER DCC status or antibody used, by a semi-quantitative H score system (range, 0 to 300) for both antibodies and in the same regions on adjacent serial sections. When a difference of H score values between the two antibodies was >50, tumors were classified as inconsistent. When the difference of H score values was <50, the tumors were considered as consistent. Overall, the mean ER and progesterone receptor (PR) status and the distribution of ER and PR levels between the inconsistent and consistent groups was very similar (see Table 1). Within the inconsistent tumor group (24 cases), 8 tumors were low ER positive (3 to 10 fmol/mg protein; 33%), 6 tumors were middle ER positive (11 to 50 fmol/mg protein; 25%), and 10 were high ER positive (>50 fmol/mg protein; 42%), as determined by ligand-binding assay. Within the consistent tumor group (39 cases), 6 cases were low ER positive (15%), 12 cases were middle ER positive (31%), and 21 were high ER positive (54%).

Table 1. Number of Tumors Expressing Detectable ER Variant in Consistent and Inconsistent Tumors

Tumors	n	ER ^{DCC}	PR ^{DCC}	D7-ER	D4-ER	D3-4-ER	D4/7-ER	D2-3/7-ER	D2-3-4-ER	D-3-7-ER	ER V. ^{OF}	ER V. ^{IF}
Consistent	39	81 (89)	60 (75)	35	9	3	4	0	0	2	6	12
Inconsistent	24	69 (86)	55 (69)	22	6	2	2	2	3	8	10	8
P				>0.05	>0.05	>0.05	>0.05	>0.05	<0.05	<0.01	<0.05	>0.05

Analysis was by χ^2 . ER V.^{OF}, out-of-frame exon-deleted ER variant mRNAs excluding D7-ER; ER V.^{IF}, in-frame exon-deleted ER variant mRNAs; ER^{DCC}, mean (SD) ER status measured by DCC assay (fmol/mg protein); PR^{DCC}, mean (SD) PR status measured by DCC assay (fmol/mg protein).

Extraction of mRNA and Reverse Transcription

For each case, a specific face of a frozen tissue block that matched the corresponding face of the paraffin block previously studied by IHA was selected.¹⁷ Total RNA was extracted from histologically defined regions within 20- μ m cryostat sections of frozen tissue using a small-scale RNA extraction protocol (Trireagent, MRCI, Cincinnati, OH) as previously described.¹⁸ Reverse transcription reactions were performed in triplicate in a final volume of 15 μ l.^{13,15} and 1 μ l of the reaction mixture was taken for subsequent PCR amplification in either long-range PCR or triple-primer PCR assays described below.

Analysis of Prevalence of ER Variant mRNAs

Prevalence of ER variant mRNAs within breast tumor samples was assessed by PCR analysis performed by a long-range PCR assay as previously described.¹⁵ The primers used consisted of 1/8U primer (5'-TGCCCTACTACCTGGAGAACG-3', sense, located in WT-ER exon 1) and 1/8L primer (5'-GCCTCCCCCGTGATGTAA-3', antisense, located in WT-ER exon 8). This primer set allowed amplification of a 1381-bp fragment corresponding to WT-ER mRNA and all deleted or inserted ER variant mRNAs containing exon 1 and exon 8 sequences. PCR amplifications were performed in a final volume of 10 μ l, in the presence of 10 nmol/L [α -³²P]dCTP (ICN Pharmaceuticals, Irvine, CA), 4 ng/ μ l each primer, and 1 U of Taq DNA polymerase (Promega, Madison, WI). Each PCR consisted of 40 cycles (1 minute at 60°C, 2 minutes at 72°C, and 1 minute at 94°C) using a thermal cycler (MJ Research PT100, Fisher Scientific, Ottawa, Canada). After PCR, 2 μ l of the reaction was denatured in 80% formamide buffer, and the PCR products were separated on 3.5% polyacrylamide gels containing 7 mol/L urea (PAGE). After electrophoresis, the gels were dried and autoradiographed for 18 hours. Identities of specific bands were then confirmed by reference to size markers, subcloning, and sequencing.¹³

Quantification of ER Clone 4 mRNA Expression

Quantification of clone 4 mRNA expression was performed using a triple-primer PCR assay as previously reported.¹⁶ Briefly, three primers, E2U (5'-AGGGTGGCAGAGAAAGAT-3', sense, located in WT-ER exon 2), E3L (5'-TCATCATTCCCCTTCGT-3', antisense, located in WT-ER exon 3), and C4L (5'-GGCTCTGTTCTGTTCCATT-3', antisense), were used during PCR, performed in the presence of [α -³²P]dCTP. These primers allowed the co-amplification of a 281-bp and a 249-bp fragment corresponding to WT-ER and clone 4 truncated ER variant mRNAs, respectively. PCR products were separated by PAGE. After electrophoresis, gels were dried and autoradiographed. Autoradiographs were analyzed with a video-densitometry system and quantitated using MCID M4 software (Imaging Research, St. Catharines, Canada). The signal corresponding to ER clone 4 was measured relative to expression of the corresponding WT-ER and expressed as a percentage relative to a reference standard (an ER-positive tumor sample) to reduce any variation due to signal intensity in different gels. ER clone 4 expression was determined from the mean of three independent RT-PCR assays performed without knowledge of the IHA status. Means obtained from the 24 IHA-inconsistent tumor samples were then compared with those found in the 39 IHA-consistent tumor samples using the Mann-Whitney rank sum test (two sided).

Results

Detection of Exon-Deleted ER Variant mRNAs within Consistent and Inconsistent Tumors

Prevalence of exon-deleted ER variant mRNAs was investigated within 63 breast tumors, previously studied by IHA using 1D5 and AER311 antibodies⁵ and subsequently classified as IHA consistent (39 cases) or IHA inconsistent (24 cases) as illustrated in

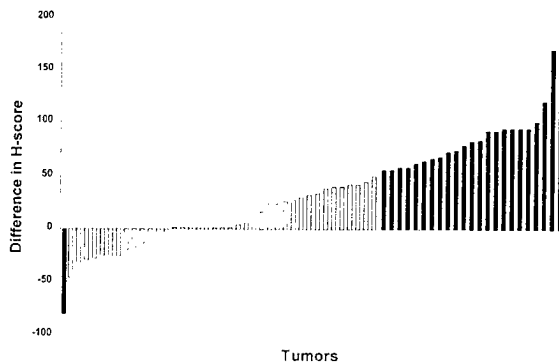


Figure 2. Graph to show the difference in H score (IHA-1D5 to IHA-311) for each of 63 tumors showing the basis for classification into IHA-consistent (< 50 H score difference; white bars) and IHA-inconsistent tumors (> 50 H score difference; black bars).

Figure 2. Long-range RT-PCR assay using primers annealing with exon 1 (1/8U) and exon 8 (1/8L) sequences first allowed assessment of the most prevalent exon-deleted variant mRNAs in comparison with the co-amplified WT-ER mRNA, as described previously.¹⁵ Several different PCR products were observed within the set of tumors studied (Figure 3) that have previously been shown to correspond to the WT-ER (1381 bp) and ER variant mRNAs deleted in exon 7 (D7-ER, 1197 bp), exon 4 (D4-ER, 1045 bp), both exons 3 and 4 (D3-4-ER, 928 bp), exons 2, 3, and 7 (D2-3/7-ER, 889 bp), both exons 4 and 7

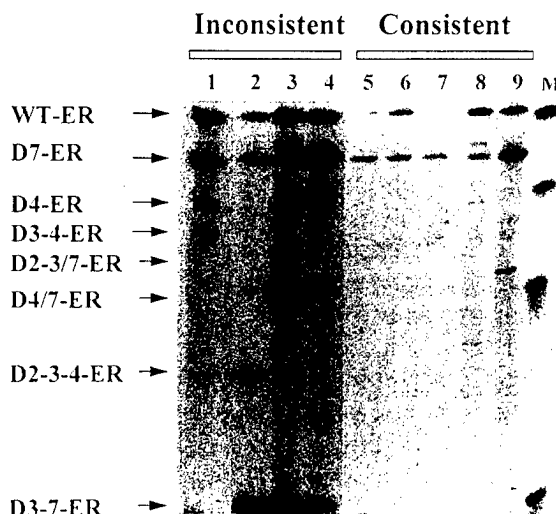


Figure 3. Comparison of exon-deleted ER variant expression between IHA-consistent¹⁵ and IHA-inconsistent^{15,16} breast tumors. Total RNA was extracted from inconsistent and consistent tumors, reverse transcribed, and subsequently amplified by PCR as described in Materials and Methods. PCR products were separated on PAGE and visualized by autoradiography. Bands migrating at 1381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were identified by isolation and sequencing as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3, and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2, 3, and 4 (D2-3-4-ER), and within exon 3 to within exon 7 (D3-7-ER), respectively. M, molecular weight marker (ϕ X174, Gibco BRL, Grand Island, NY).

(D4/7-ER, 861 bp), exons 2, 3, and 4 (D2-3-4-ER, 737 bp), and within exon 3 to within exon 7 (D3-7-ER, 580 bp), respectively.¹⁵ Results obtained for IHA-consistent and IHA-inconsistent tumor subgroups are summarized in Table 1. D7-ER, D4-ER, D3-4-ER, and D4/7-ER variant mRNAs were detected at the same frequency in both subgroups. However, D2-3/7-ER, D2-3-4-ER, and D3-7-ER mRNAs were preferentially detected in IHA-inconsistent tumors. This increased prevalence reached statistical significance for both D2-3-4-ER and D3-7-ER mRNAs ($P < 0.05$ and $P < 0.01$). Given that the D7-ER variant was detected uniformly (>90%) in both subgroups, we chose to assess the remainder of the variant mRNAs that were not uniformly detected (ie, all variants except D7-ER). These were then considered with respect to the putative ER-like protein they should encode and classified further into two subgroups. In-frame variants (ER V.^{IF}) comprised those with a sequence modification that did not introduce a shift in the reading frame and that could encode proteins theoretically recognized by both 1D5 and AER-311 antibodies (D4-ER and D3-4-ER variant mRNAs). Out-of-frame variants (ER V.^{OF}) comprised variants encoding proteins theoretically only recognized by 1D5 antibody (D4/7-ER, D2-3/7-ER, D2-3-4-ER, and D3-7-ER). ER V.^{IF} were detected in 12 (31%) and 8 (33%) IHA-consistent and IHA-inconsistent tumors, respectively. At the same time, ER V.^{OF} were detected in only 6 (15%) IHA-consistent compared with 10 (42%) IHA-inconsistent tumors, respectively ($P < 0.05$, χ^2 analysis).

Quantification of Clone 4 mRNA Expression

Expression of a prevalent truncated ER mRNA variant, the ER-clone 4 variant, which is also suspected to encode a truncated ER-like protein, was then analyzed by triple-primer RT-PCR using three primers to allow the co-amplification of WT-ER mRNA together with clone 4 variant mRNA, as described previously.¹⁶ Typical results from IHA-consistent and IHA-inconsistent tumors are shown Figure 4. PCR products (bands of 281 bp and 249 bp) corresponding to WT-ER and ER clone 4 mRNAs were observed in all tumors. Using the Mann-Whitney rank sum test (two sided), the relative expression of clone 4 truncated variant ER mRNA to WT-ER mRNA was also found to be significantly ($P < 0.01$) higher in IHA-inconsistent tumors (median = 80.4%, SD = 18.7%) versus IHA-consistent tumors (median = 62.4%, SD = 14.4%; Figure 5)

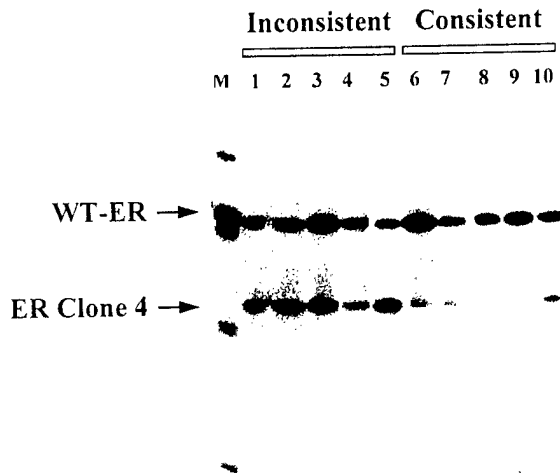


Figure 4. Expression of clone 4 variant ER mRNA in tumors representative of IHA-inconsistent (lanes 1 to 5) and IHA-consistent (lanes 6 to 10) tumor subgroups. RNA extracted from tumors was analyzed by triple-primer PCR as described above. Upper and lower arrows show wild-type and clone 4 corresponding signals, respectively.

Discussion

Using PCR-based approaches that allow the investigation of the prevalence of different exon-deleted and truncated ER variant mRNAs within breast tumor samples, we have investigated ER variant mRNA expression within 63 breast tumors that presented similar (IHA-consistent) or different (IHA-inconsistent) results when assessed for ER expression by IHA performed with an antibody (1D5) recognizing the amino terminal as compared with an antibody

(AER311) targeting the carboxy terminal of the ER protein. We have found that, whereas variants such as D7-ER, D4-ER, D3-4/ER, and D4/7-ER are detected at the same frequency in IHA-inconsistent and IHA-consistent breast tumors, other variants, including D2-3/7-deleted, D2-3-4-ER, and D-3-7-ER, are preferentially detected in IHA-inconsistent cases. This difference between subgroups was statistically significant for two of these variants: exon-2-3-4-deleted ER and exon-3-7-deleted ER. Both of these two variant mRNAs possess sequence modifications that introduce a shift in the WT-ER coding sequence that would encode ER-like proteins containing the amino-terminal TAF-1 transactivation domain but missing all the carboxyl-terminal extremity of WT-ER protein (Figure 1). These putative variant ER proteins would therefore theoretically be recognized by 1D5 antibody but not AER311 antibody. Furthermore, detectable expression of the subset of variant mRNAs able to encode truncated ER-like proteins (except the uniformly prevalent D7-ER variant that was detected in all but 6 tumors of the 63 studied) was significantly higher in the IHA-inconsistent tumor group. In contrast, detectable expression of variants encoding in-frame proteins that should be recognized by both antibodies was no different between tumor subgroups. Taken together, these results are in keeping with the hypothesis that ER variant mRNAs encoding truncated ER proteins may participate in the synthesis of ER-like proteins differentially recognized by 1D5 and AER311 antibodies. This assumption is also further supported by the results obtained using a quantitative PCR-based approach applied to the same tumors, which indicate that IHA-inconsistent tumors also possess significantly higher levels of ER clone 4 truncated variant relative to WT-ER compared with IHA-consistent tumors.

Until the development of antibodies that are specific for individual ER variant proteins, the premise that proteins encoded by ER variant mRNAs may directly interfere with ER immunodetection and determination of ER status by IHA remains to be proven. It is clear from *in vitro* laboratory studies that ER variants can encode proteins that possess a variety of dominant negative, positive, or undetectable activities when tested for their ability to interfere with transactivation of classical ER enhancer sequences/elements.^{6-9,19} Thus, although we and others^{4,5} have observed a relative increase in amino-terminal signal that may correspond to increased truncated ER proteins, the functional implications in terms of response to endocrine therapy will depend

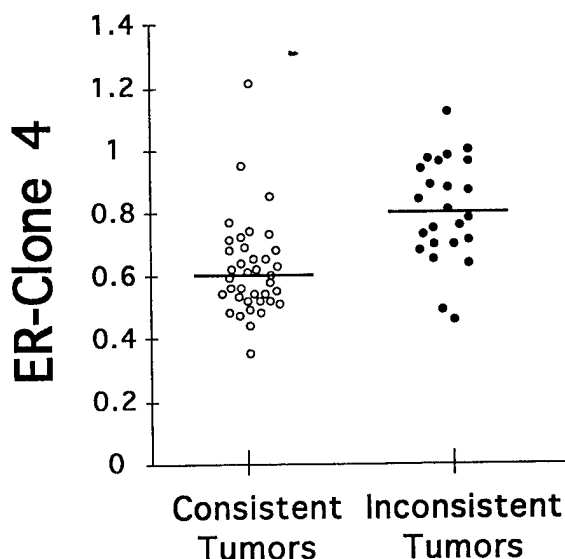


Figure 5. Comparison of the relative expression of ER clone 4 variant mRNA in IHA-inconsistent breast tumors and in IHA-consistent breast tumors. For each sample, the mean of three independent measures of clone 4 expression was expressed as a percentage of the corresponding wild-type ER signal. The difference between two groups is statistically significant ($P < 0.01$, Mann-Whitney rank sum test, two sided).

on the nature of the specific ER variant activities in a given tumor.

Although a good correlation between ER-DCC and ER-IHA is often found, approximately 20% of cases are discordant.⁵ It is believed that the cause of this discordance is multifactorial and both ER-DCC-positive/IHA-negative and ER-DCC-negative/IHA-positive cases have been attributed overall to tumor heterogeneity, sampling, variable frozen tissue handling, and formalin fixation.²⁰ However, an explanation for discordant results is not always apparent in specific cases.²⁰ Thus, although recent studies have shown that immunodetection using 1D5-IHA can accurately predict endocrine response of breast cancer,²¹ the relative predictive value of ER-DCC versus ER-IHA is still under debate.²⁰⁻²³ In the light of our results, and laboratory evidence to suggest that ER variant proteins encoded by ER variant mRNAs may participate in endocrine response,⁶⁻¹⁴ it may be important to assess ER variant expression in future studies concerning ER-IHA status and response to endocrine therapy.

Interestingly, the two exon-deleted ER variant mRNAs, the expression of which was shown here to be correlated to inconsistent results by IHA (ie, D2-3-4-ER and D-3-7-ER), have not been detected until recently.¹⁵ However, our previous studies⁵ indicate that expression of these variants may be associated with high-grade tumors and high ER level, respectively.¹⁵ Similarly, we have shown that a higher level of ER clone 4 mRNA expression correlates with tumor progression and poor prognosis.^{16,24} This suggests not only that these ER variant mRNAs may contribute to discrepant IHA results but also that alteration of their expression is associated with tumor progression.

In conclusion, we have found a significant correlation between expression of certain ER variant mRNAs and inconsistent IHA results after assessment and comparison of ER expression with antibodies directed to either amino- or carboxyl-terminal epitopes in human breast cancer. These data add to the growing body of evidence that suggests that ER variants may be translated *in vivo* into ER-like proteins.^{5,25,26} Finally, these results suggest that ER variant expression may be an important parameter to consider in the determination of ER status in human breast cancer.

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APPENDIX 22

Leygue E, Dotzlaw H, Lu B, Glor, C., Watson PH and Murphy LC

Estrogen receptor beta: mine is longer than yours?

J Clin Endocrinol Metab, 83:3754-3755, 1998.

LETTERS TO THE EDITOR

Estrogen Receptor β : Mine Is Longer Than Yours?^a

To the editor:

Two years after the cloning of the second estrogen receptor, ER- β , the primary sequence encoding the N-terminus of the protein still remains uncertain. The demonstrated importance of estrogen signalling in normal and abnormal development of multiple tissues justifies characterization of this region.

Estrogens, involved in the normal development of a wide tissue spectrum including breast, uterus, brain, and bone, are also implicated in several diseases such as breast and endometrial cancers and osteo-

base upstream of the start codon. This extra base alters frame and removes the previously observed in-frame stop codon, resulting in a cDNA that could encode 64 additional N-terminal amino-acids (R2, Fig. 1). In May 1998, two sequences submitted to Genbank extended the initial 5'-extremity of the mER- β cDNA. The encoded mER- β proteins contain 45 and 64 extra amino-acids N-terminal to M1 (M2 and M3, Fig. 1), respectively. All three species present strong sequence similarities in this N-terminal region, although the rodent open reading frames are 19 amino-acids longer than the human.

Altogether, these observations raise important questions. Are cloning strategies and/or tissue/species-specific expression of different forms of

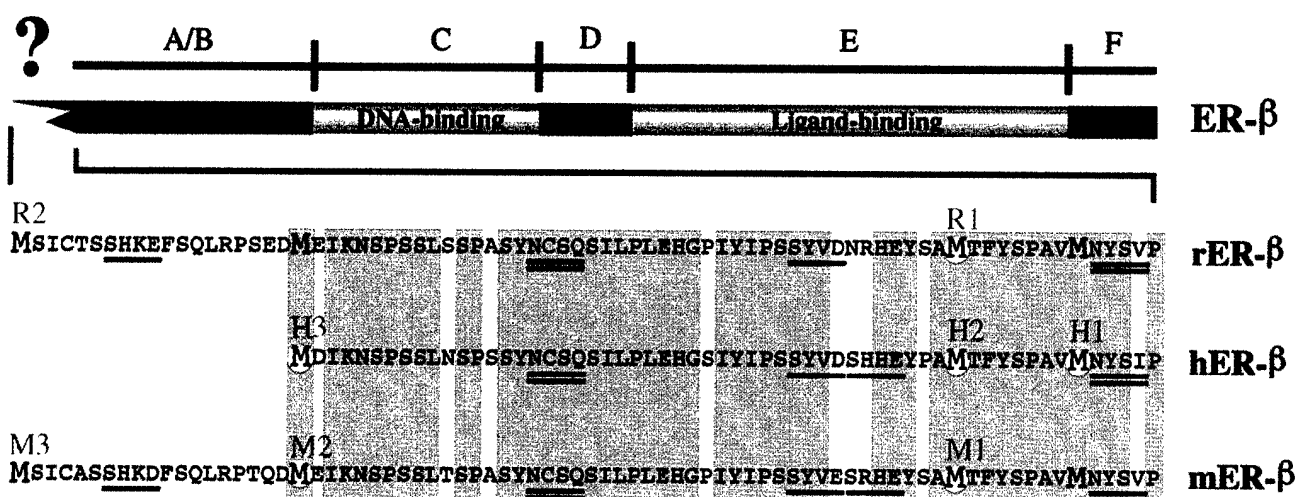


FIG. 1. Schematic representation of rat, human, and mouse ER- β protein. The amino acid composition of N-terminal extremities of ER- β proteins predicted from cloned cDNAs is shown. For each species (rat R, human H, mouse M), initiating methionine codons are indicated in their chronological order of identification (1, 2, 3). Similarities between the three species are indicated by blue boxes. Sequences underlined by a single and double line represent putative phosphorylation and glycosylation motifs, respectively. Genbank accession numbers of sequences encoding R1, R2, H1, H3, M1, M2, and M3 are U57439, AJ002602, X99101, AB006590, U81451, AF063853, and AF067422, respectively.

porosis. In 1995, the cloning (1) of ER- β from a rat prostate cDNA library, led to the need to fully re-evaluate estrogen signalling in target tissues. Like estrogen receptor α (2), rER- β is a ligand-dependent transcription factor that binds estrogen and antiestrogen. Relying on the presence of an in-frame stop codon upstream of their coding sequence, Kuiper *et al.* (1) considered their open reading frame to encode the full length protein (R1, Fig. 1). Shortly thereafter, a human homologue, hER- β , was cloned (3) from testis (H1, Fig. 1). This cDNA encoded a protein 8 amino-acids shorter than rER- β . In 1997, the hER- β cDNA sequence was extended (4) to a start codon corresponding to the first rat methionine codon (H2, Fig. 1), and the mouse homologue, mER- β , was cloned (5) (M1, Fig. 1).

In 1998, following cDNA library screening and/or polymerase chain reaction, the N-terminus of ER- β was extended in all three species. Ogawa *et al.* (6) isolated from human testis a hER- β cDNA that could encode 45 additional amino-acids (H3, Fig. 1). This sequence plus the presence of an upstream in-frame stop codon was recently confirmed by Moore *et al.* (7). In April 1998, a prostate rER- β cDNA sequence was submitted to Genbank, that differs from the initial sequence by only one

ER- β responsible for observed discrepancies? Do longer forms of ER- β , as yet unidentified, exist? If so, how is expression regulated? What are the functions associated with these extra amino-acids? The presence of putative phosphorylation and glycosylation motifs within these additional amino-acid sequences suggest possible regulation of putative function(s). Moreover, because of the demonstrated importance of the N-terminal region in ER- α , particularly the involvement of the AF-1 domain in hormone independent activation of the receptor (8), the elucidation of the function(s) associated with these new regions is necessary. The majority of functional studies undertaken to date were performed with constructs lacking these N-terminal amino-acids: addressing the above questions is critical to fully understanding the involvement of ER- β in estrogen signalling.

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APPENDIX 23

Murphy LC, Dotzlaw H, **Leygue E**, Coutts, A., and Watson PH

The pathophysiological role of estrogen receptor variants in human breast.

J Steroid Biochem Mol Biol, 65:175-180, 1998.



The Pathophysiological Role of Estrogen Receptor Variants in Human Breast Cancer

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The accumulated evidence supports the expression of estrogen receptor variants at both the mRNA and protein levels. The relative level of expression of some estrogen receptor variant mRNAs and possibly progesterone receptor variant mRNAs is altered during breast tumorigenesis and breast cancer progression. The altered expression of estrogen receptor variants may effect estrogen signal transduction as well as the interpretation of assays where the estimation of estrogen receptor levels is used as a guide to treatment strategies and prognosis. © 1998 Elsevier Science Ltd. All rights reserved.

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INTRODUCTION

The estrogen receptor is considered pivotal in the mechanism by which estrogen interacts with its target cells and mediates its specific effects. Classically, the estrogen receptor is considered to be a ligand activated transcription factor, which upon estrogen binding undergoes conformational changes which allow it to dimerize, tightly bind to estrogen responsive DNA sequences and alter transcription of target genes [1, 2]. However, the diverse effects of estrogen on target tissues [3–6] and the observation that many human breast cancers develop estrogen independence despite the continued expression of the estrogen receptor [7] suggest that the concept of estrogen action described above is unlikely to simply explain all aspects of estrogen action [2, 8]. Evidence has accumulated over the last decade supporting the existence of estrogen receptor variants [9, 10]. Therefore the possibility exists that estrogen receptor variants may have a pathophysiological role in estrogen action. For example, the pattern of estrogen receptor variant expression may influence which set of estrogen responsive genes are transcribed. The following discussion reviews the evidence available to support a pathophysiological

role of estrogen receptor variants in human breast tissues.

STRUCTURE OF ESTROGEN RECEPTOR VARIANT MRNAS

Most data supporting the existence of estrogen receptor (ER) variants have been at the mRNA level. Two main structural patterns of estrogen receptor variant mRNAs have been consistently identified: the truncated ER mRNAs [11] and the deleted ER mRNAs [12]. Using a long range reverse transcription-polymerase chain reaction analysis (RT-PCR) [13] which detects all deleted ER variant mRNAs at a frequency relative to their initial mRNA representation in the unamplified sample [14], the most frequently detected and likely the most abundant deleted ER variant mRNAs in human breast tumors appear to be the exon 7 deleted ER mRNA [13, 15] and the exon 4 deleted ER mRNA [13, 16]. However, in some breast tumors other deletion variants such as an exon 3 + 4 deleted ER mRNA [13] and an exon 4 + 7 deleted ER mRNA [13] have been frequently detected. An exon 5 deleted ER variant mRNA is rarely detected using the long range RT-PCR approach, suggesting that its abundance is low compared to several other ER deleted mRNAs. However, when specific PCR primers are used to measure only the exon 5 deleted ER mRNA relative to the wild-type ER mRNA, the levels of the exon 5 variant mRNA are found to vary

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amongst breast tumor samples [17] as well as between normal breast tissues and breast tumors [18]. Also truncated ER variant mRNAs have been frequently detected at relatively high abundance in several human breast tumors [19,20]. In fact the truncated ER variant mRNAs are the only ER variant tran-

scripts that have been detected by Northern blotting analysis and the entire cDNAs cloned and sequenced [11,19]. A commonly expressed truncated ER mRNA is the clone 4 truncated ER mRNA. The predicted proteins of these relatively most abundant ER variant transcripts are shown schematically in

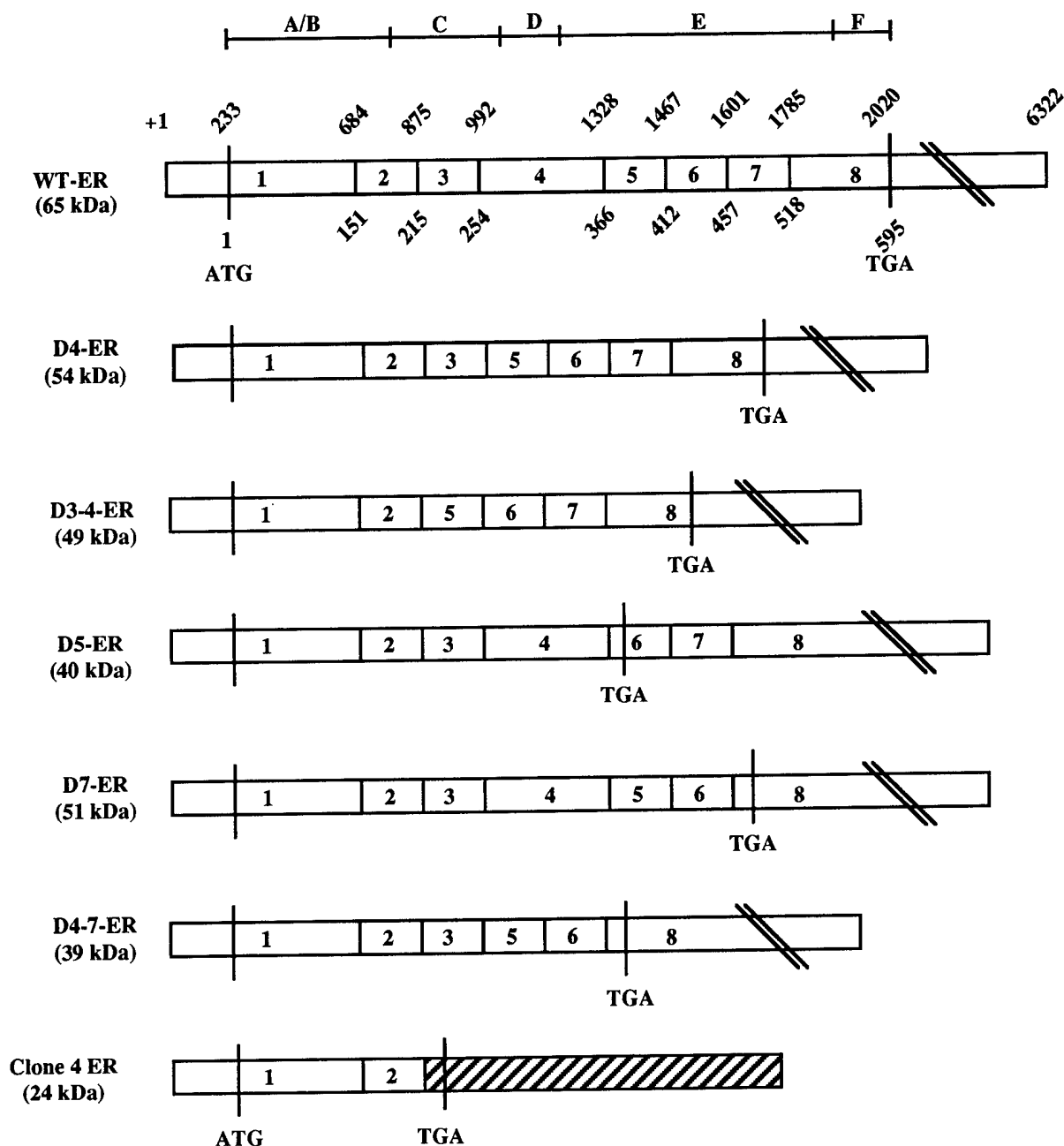


Fig. 1. Schematic diagram of the wild-type human estrogen receptor (ER) cDNA, which contains 8 different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another trans-activating function (AF-2). The numbering on the top of the cDNA refers to the nucleotide position as defined in reference [39]. Below the wild-type ER cDNA are the various putative exon deleted and truncated ER cDNAs. ATG shows the translation initiation codons, TGA shows the inframe translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in Ref. [39]. D = deletion, and the estimated molecular mass (kDa = kiloDaltons) of each open reading frame is shown in brackets. Molecular masses were estimated using MacVector version 4.1.4 software.

Fig. 1. All of these variant transcripts will encode ER proteins missing some structural/functional domains of the wild-type ER. In many cases i.e. the exon 7 deleted, the exon 4 + 7 deleted and the clone 4 truncated ER variant mRNAs will encode C-terminally truncated proteins in which the ligand binding domain, the ligand dependent transcriptional activity (AF-2) and the ligand dependent dimerization domain are significantly impaired. Although the exon 4 deleted and the exon 3 + 4 deleted transcripts are inframe, studies indicate that the proteins encoded by these transcripts cannot bind ligand and have little, if any, ligand dependent transcriptional activity [16, 21]. Furthermore, the proteins encoded by the clone 4 truncated, the exon 3 deleted, the exon 3 + 4 deleted and the exon 4 + 7 deleted variant mRNAs are all unlikely to bind significantly to classical estrogen responsive DNA sequences. A common feature of all the relatively abundantly expressed ER variant mRNAs described above, is they would encode proteins with intact A/B regions. This region has been described to contain a promoter and cell-type specific

transcriptional activity [22, 23], although its ability to function in the complete absence of an intact DNA binding domain is unexplored. In summary, a large body of molecular data exists to support the potential expression of ER variant proteins.

SPECIFICITY OF ESTROGEN RECEPTOR VARIANT EXPRESSION

The available studies provide evidence for an extensive and complex pattern of alternative splicing associated with the estrogen receptor gene, which appears to be altered during breast tumorigenesis. It has been suggested that the complex pattern of exon deleted ER variant mRNA expression is specific for the estrogen receptor, since similar variants for the glucocorticoid receptor and the retinoic acid receptors alpha and gamma have not been found in breast tumor tissues [24]. We have also investigated the pattern of exon deleted variant mRNA expression in breast tumors using a long range RT-PCR approach [13] for the progesterone receptor (PR) [25],

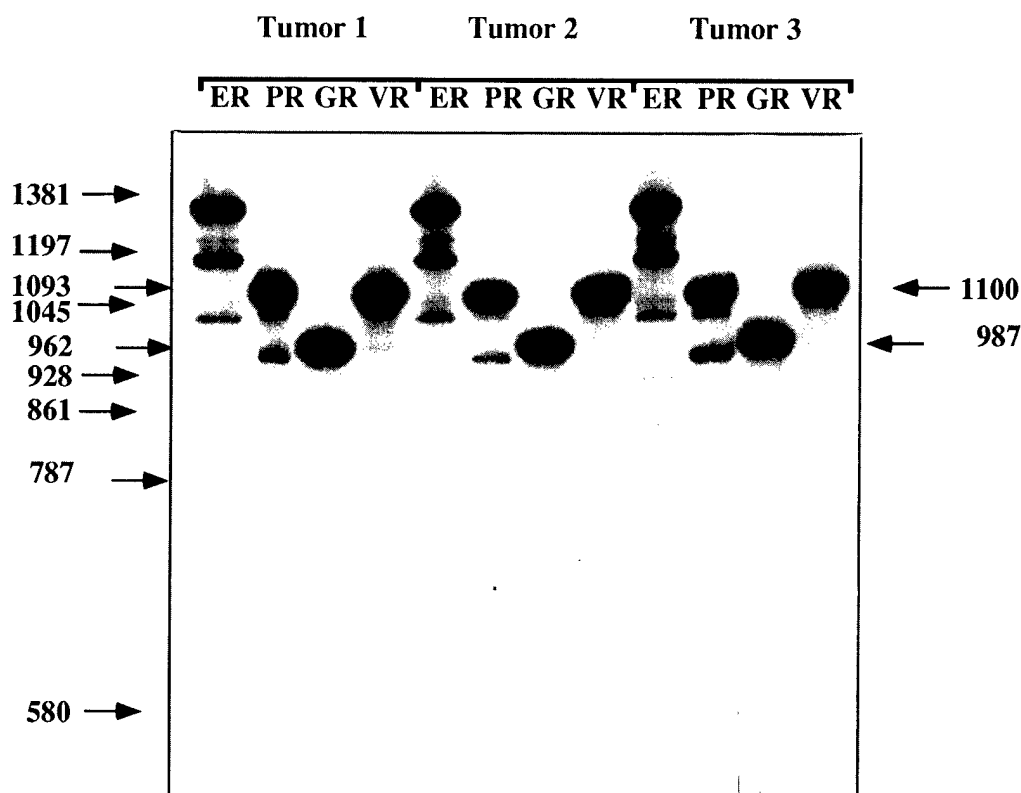


Fig. 2. Autoradiograph of long range RT-PCR analysis for ER, PR, GR and VR mRNA isolated from three human breast tumor biopsy samples. All tumors were ER+ and PR+ by ligand binding assay. The primers and RT-PCR conditions for ER and PR were as previously described [13, 25] except that the annealing time was 30 seconds only. The primers for the GR are as previously described [24] and located in exons 2 and 8, respectively. The RT-PCR conditions were those described above for ER and PR. The primers for the VR are: VR-U 5'-GAAGCGGAAGGCACTAT-3', sense 155-171 as defined in [27]; VR-L 5'-GAGCACAAAGGGCGTTA-3', antisense 1240-1256 as defined in Ref. [27]. D = deletion. The numbers beside the arrows represent the sizes (bp) of the PCR product: 1381 is WT-ER; 1197 is D7-ER; 1100 is WT-VR; 1093 is WT-PR; 1045 is D4-ER; 987 is WT-GR; 962 is D6-PR; 928 is D3 + 4-ER; 861 is D4 + 7 ER; 787 is D4-PR; 580 is D3 + 7-ER [13, 25].

the glucocorticoid receptor (GR) [26] and the vitamin D3 receptor (VR) [27]. Our data, shown in Fig. 2, demonstrate that little, if any, deleted variant mRNAs for the GR and VR were detected in the three breast tumors examined. However, in these same breast tumors, several exon deleted variant mRNA species for both the ER and PR were abundantly expressed (Fig. 2). Importantly, our published data have shown that the expression of the exon 5 deleted ER variant mRNA and the truncated clone 4 ER variant mRNA is elevated in breast tumors compared to normal breast tissues [18,28] and our preliminary data suggest that the level of the exon 6 deleted PR variant mRNA was more highly expressed relative to the wild-type PR mRNA in breast tumors compared to normal breast tissues (Leygue *et al.* unpublished data). These data suggest that the mechanisms generating alternatively spliced forms of both ER and PR are unlikely to be due to a generalized deregulation and/or alteration of splicing processes within breast tumors. They also suggest that the mechanism(s) is specific for the sex steroid hormone receptor genes and the alterations seen in breast tumors may have a role in altered actions of estrogens and progestins in human breast tumorigenesis.

IDENTIFICATION OF ESTROGEN RECEPTOR VARIANT PROTEINS

Recent data published from several independent groups strongly support the detection of estrogen receptor like proteins which could correspond to some of the previously identified estrogen receptor variant mRNAs in both cell lines and tissues *in vivo*.

An ER-like protein consistent with that predicted to be encoded by the exon 5 deleted ER transcript is expressed naturally in some BT 20 human breast cancer cell lines [29]. A monoclonal antibody specific to the predicted unique C-terminal amino acids of an exon 5 deleted ER protein was developed, and used to demonstrate immunohistochemically the presence of this protein in several human breast cancer samples [30]. An exon 4 deleted ER variant mRNA has been identified in both normal and neoplastic ovarian tissue [31]. Western blotting analyses revealed the presence of the expected 65 kDa wild-type ER protein as well as a 53 kDa protein which was recognized by ER antibodies to epitopes in the N-terminus and C-terminus of the wild-type protein, but not with an antibody recognizing an epitope encoded by exon 4 [31]. These data strongly support the hypothesis that the small sized ER-like protein in the ovarian tissue extracts was encoded by an exon 4 deleted ER variant mRNA.

Recently, we have analyzed several human breast tumors immunohistochemically for ER expression, comparing side-by-side an antibody recognizing an N-terminally localized epitope in the wild-type ER

protein (1D5), and an antibody recognizing a C-terminally localized epitope in the wild-type ER protein (AER311). It was found that although in many tumors the immunohistochemical results using each antibody showed good concordance with each other, in some tumors the results were discordant, with the signal tending to be higher with the N-terminal antibody. Since many of the proteins predicted from variant ER mRNAs would be truncated at the C-terminus and not contain the epitope recognized by the C-terminal antibody, one interpretation of these data would be that truncated variant ER proteins are more highly expressed in the discordant group of tumors [32]. This hypothesis was tested by investigating the pattern and relative expression of variant ER mRNAs in the discordant and concordant groups of breast tumors. Several ER variant mRNAs (the clone 4 truncated ER mRNA, the exon 2, 3 + 7 deleted ER mRNA, the exon 2, 3 + 4 deleted ER mRNA and the variant deleted within exon 3 to within exon 7) which encode putative truncated ER-like proteins that would be recognized only by an N-terminally targeted antibody were preferentially and more highly expressed in the discordant breast tumor group [33]. While this indirect approach does not specifically identify ER variant proteins, the data suggest that the ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER measured by immunodetection assays using N- or C-terminal antibodies.

The accumulated data provide strong support for the ability of, at least some ER variant mRNAs to be stably translated into proteins detectable by conventional methodologies. Such proteins may have a functional role in altered estrogen signal transduction in human breast tumorigenesis.

FUNCTIONAL SIGNIFICANCE OF ESTROGEN RECEPTOR VARIANT EXPRESSION

The important question to be addressed now is the role of ER variants in those tissues in which they are expressed. One approach has been to recombinantly express individual ER variants, alone and together with the wild-type ER, and determine effects on transcriptional activity as measured by a classical estrogen response element (ERE)-reporter gene. This has demonstrated the exon 5 deleted ER to have constitutive activity [34] and wild-type ER inhibitory activity [30]. As well the exon 3 deleted ER [35], the exon 4 deleted ER [31] and the exon 7 deleted ER [15] have been identified in some systems to have wild-type ER inhibitory activity. Some ER variants such as the exon 3 + 4 deleted variant have been shown to enhance wild-type ER activity, at least at the basal level [36]. Other ER variants such as the truncated clone 4 ER have no detectable activity in similar assays [11].

Another approach has been to determine if the ER variants are differentially expressed in normal vs neoplastic breast tissues. The accumulated data support the increased expression of the exon 5 deleted [18, 31] and the clone 4 truncated ER mRNAs [28] in breast tumors with good prognostic features compared to normal breast tissues. Furthermore it has been suggested that decreased expression of an exon 3 deleted ER mRNA occurs in breast tumors compared to normal breast tissue [37]. Further changes seem to occur during the later stages of breast cancer progression since the relative level of expression of the clone 4 truncated ER mRNA was found significantly elevated in breast tumors with characteristics of poor prognosis and endocrine resistance vs those with characteristics of good prognosis and endocrine sensitivity [20]. Moreover, increased expression of an exon 3 + 4 deleted ER was found associated with the estrogen independent, ER positive phenotype in a breast cancer cell line model [36] and preliminary data support the functional involvement of the over-expressed ER variant in the estrogen independent phenotype (Coutts *et al.*, unpublished data).

Inconsistent results have been obtained with respect to stably overexpressing an individual ER variant in a hormone dependent breast cancer cell line and the resulting development of endocrine resistance [10, 38]. The data suggest that altered ER variant expression may contribute to altered estrogen receptor activity which together with other factors will contribute to breast cancer progression and the eventual development of hormone independence and resistance to endocrine therapy [7].

CONCLUSIONS

Estrogen receptor variants can be detected at both the mRNA and protein levels. The level of expression of some ER variants is altered during breast tumorigenesis and breast cancer progression. The functional involvement of ER variants and possibly PR variants in breast cancer progression and altered responses to these steroid hormones requires further detailed investigation. More immediately, the impact of expression of variant ER and PR on the determination of ER and PR immunohistochemically as markers of prognosis and treatment response in breast cancer requires assessment.

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APPENDIX 24

Murphy LC, **Leygue E**, Dotzlaw H, Coutts AS, Lu B, Huang A, Watson PH

Multiple facets of the estrogen receptor in human breast cancer.

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Multiple Facets of the Estrogen Receptor in Human Breast Cancer.

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Abstract. The potential for multiforms of estrogen receptor like proteins to be expressed in human breast tissue is supported by the detection of estrogen receptor- α mRNA, estrogen receptor- β mRNA and their variant mRNAs in both normal and neoplastic human breast tissues. This suggests that studies of the interaction of estrogen and antiestrogen with human breast tissue must take into account the multifaceted nature of the estrogen receptor. Alterations in the expression of the mRNA of the various estrogen receptor facets during breast tumorigenesis and breast cancer progression suggest possible roles in these complex processes. However, there are still major gaps that need to be addressed before we have a clear idea of the pathophysiological and functional relevance of the experimental results so far published.

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Introduction.

Estrogen is a major regulator of mammary gland development and function as well as affecting the growth and progression of mammary cancers (1,2). In particular the growth responsiveness of breast cancer cells to estrogen is the basic rationale for the efficacy of the so-called endocrine therapies such as antiestrogens. Estrogens mediate their action via the estrogen receptor (ER) which belongs to the steroid/thyroid/retinoid receptor gene superfamily (3). The protein products of this family are intracellular ligand-activated transcription factors regulating the expression of several gene products, which ultimately elicit a target tissue specific response (4). Indeed ER together with progesterone receptor (PR) expression in human breast tumors are important prognostic indicators as well as markers of responsiveness to endocrine therapies (5,6). However, although the majority of human breast cancers are thought to be initially hormone responsive it is well appreciated that alterations in responsiveness to estrogen must occur during breast tumorigenesis and in particular during breast cancer progression since several ER+ breast cancers are *de novo* resistant to endocrine therapies and of those that originally respond many develop resistance. This progression from hormonal dependence to independence, is a significant clinical problem since it limits the usefulness of the relatively non-toxic endocrine therapies and is associated with a more aggressive disease phenotype (7). This occurs despite the continued expression of ER and often PR (8,9). The ER is pivotal in estrogen and antiestrogen action in any target cell, however, the nature of the ER is now clearly multifaceted.

Until recently it was thought that only one ER gene existed. However a novel ER, now referred to as ER- β , has recently been cloned and characterized (10,11). Moreover, it has recently been shown that ER- β mRNA is expressed in both normal and neoplastic human breast tissue (12-14). This suggests that ER- β may have a role in estrogen action in both normal and neoplastic human breast tissue. Furthermore, it has now become apparent that several variant mRNA species of both the classical ER- α and ER- β can be expressed in human breast tissues and may therefore have roles in estrogen and antiestrogen signal transduction (13,15-18). The current data suggest that an evaluation of estrogen interaction with human breast tissue needs to include ER- α , ER- β and any variant forms of these receptors that may be

expressed. The following article focuses on this multifaceted nature of the ER in human breast tissues.

I. Estrogen Receptor- α and its Variants.

i. **Identification of ER- α variant mRNAs in Human Breast Tissues.** A large body of data has accumulated supporting the existence of ER- α variants (19,20). The majority of the data supporting the expression of ER- α variants has been at the mRNA level. Two main structural patterns of ER- α variant mRNAs have been consistently identified: the truncated ER- α mRNAs (21) and the exon deleted ER- α mRNAs (22). The truncated ER- α mRNAs were originally identified by Northern blot analysis as fairly abundant smaller sized mRNA species in some human breast cancer biopsy samples (23). The cDNAs of several truncated ER- α mRNAs have been cloned and found to contain authentic polyadenylation signals followed by poly A tails. The exon deleted ER- α mRNAs have been identified mainly from reverse transcription polymerase chain reaction (RT-PCR) products using targeted primers.

Multiple ER- α variant mRNAs are often detected in any one tumor sample. In order to determine the relative frequency and pattern of variant expression in any one sample an RT-PCR approach was developed which allowed the simultaneous detection of all deleted ER- α variant mRNAs containing the primer annealing sites in exons 1 and 8, at levels that represent their initial relative representation in the RNA extract. Since truncated transcripts do not have exon 8 sequences they will not be measured by this technique. Examples of the results obtained are shown (Figure 1) and serve to illustrate: i) that a complex pattern of exon deleted variant ER- α transcripts are expressed in any one tumor; ii) that the pattern and relative frequency of detection of ER- α variant mRNAs can vary between tumors, and iii) that in some cases the relative frequency of detection of individual ER- α variant mRNAs can be correlated with known prognostic markers (24).

An example of such a correlation is shown in Figure 2 (25). The expression of the truncated clone 4 ER- α variant mRNA was measured relative to the wild type ER- α mRNA in a group of breast tumors. The relative expression of the clone 4 variant was significantly increased in those tumors with characteristics of poor prognosis compared to those tumors with

good prognostic characteristics i.e. clone 4 expression was higher in large tumors with high S phase fraction and from patients with nodal involvement; compared to small tumors with low S phase fraction and from patients without nodal involvement. As well in this group the relative expression of clone 4 was significantly higher in PR negative tumors versus PR+ tumors, suggesting a correlation of increased truncated variant expression and markers of endocrine resistance.

The data supported the possibility that ER- α variant proteins might exist, their pattern and frequency changed amongst tumors, and in some cases the expression of single ER- α variant mRNA species was correlated with known markers of prognosis and endocrine sensitivity. Which in turn suggested the hypothesis that altered expression of ER- α variants could be a mechanism associated with hormonal progression in breast cancer.

ii. Putative Biological Significance of ER- α variant mRNAs. Important issues associated with the biological significance of ER- α variant mRNAs are whether they are tumor specific and the presence of proteins corresponding to those predicted from the variant mRNAs.

iiia. The Expression of ER- α variant mRNAs in Normal and Neoplastic Human Breast Tissue. Most studies investigating ER- α variant mRNAs have used human breast cancer tissues or cell lines (19). However, it is now known that both truncated and exon deleted ER- α variant mRNAs can be detected in other tissues including normal, non-neoplastic tissues (19). In particular ER- α variant mRNAs have been identified in normal human breast tissue and cells (26-29). Therefore, ER- α variant mRNAs are not tumor specific, are not found in the complete absence of the wild type ER- α mRNA and are most likely generated by alternative splicing mechanisms.

This raised the question of whether the expression of ER- α variant mRNAs is altered during breast tumorigenesis and/or progression. When the level of expression of individual variant ER- α mRNAs was measured relative to the level of the wild type ER transcript differences between normal and breast tumor tissues were found. The relative expression of clone 4 truncated ER- α variant mRNA and the exon 5 deleted ER- α variant mRNA but not the

exon 7 deleted ER- α variant mRNA was significantly increased in breast tumors compared to normal breast tissues obtained from both reduction mammoplasties and normal tissues adjacent to breast tumors (26,27). Preliminary data suggests that this is also true for samples of ER positive breast tumors and their matched, adjacent normal tissues (Leygue *et al.*, unpublished data). As well, there is evidence suggesting that an exon 3 deleted ER- α variant mRNA is decreased in breast cancers compared to normal human breast epithelium (29). Since this ER- α variant mRNA encodes a protein which can inhibit wild type ER- α transcriptional activity (30) and cause growth suppression when stably overexpressed in ER+ MCF-7 human breast cancer cells (29), it was concluded that the exon 3 deleted ER- α variant may function to attenuate estrogenic effects in normal mammary epithelium, a function markedly reduced via decreased exon 3 deleted ER- α expression during breast tumorigenesis. In preliminary studies of ER+ human breast tumor samples and their matched adjacent normal tissues, a statistically significant decreased relative expression of the exon 3 deleted ER- α mRNA in the tumor compared to the normal breast tissues was noted (Leygue *et al.*, unpublished data).

The available data provide evidence for an extensive and complex pattern of alternative splicing associated with the ER- α gene, which may be altered during breast tumorigenesis.

iib. Specificity of ER- α Splice Variants in Human Breast Tumors. It is unlikely that the mechanisms generating alternatively spliced forms of ER- α are due to a generalized deregulation of splicing processes within breast tumors, since similar variants for the glucocorticoid receptor (16,28), the retinoic acid receptors- α and γ (28) and vitamin D3 receptor (16) have not been found in breast tumor tissues. However, similar splice variants of PR (section 3) were found in both normal and neoplastic breast tissues (31,32).

iic. Expression of ER- α Variant mRNAs During Breast Cancer Progression. As described above the relative expression of at least one ER- α variant mRNA i.e. clone 4 truncated ER- α mRNA, is significantly higher in primary breast tumors with characteristics of poor prognosis (including the presence of concurrent lymph node metastases) compared to primary tumors with good prognostic markers (including lack of concurrent lymph node

metastases) (25). An increased relative expression of exon 5 deleted ER- α mRNA has been found in locoregional breast cancer relapse tissue (in the same breast as the original primary tumor but no lymph node metastases) obtained from patients following a median disease-free interval of 15 months, compared to both the corresponding primary breast tumor (33), and the primary breast tumor tissue of patients who did not relapse during this period. Although the difference did not reach statistical significance, these same authors reported a trend towards higher relative expression of exon 5 deleted ER- α mRNA in primary tumors of women who relapsed compared to primary tumors of those that did not relapse. Together these data suggest that as well as altered expression of ER- α variant mRNA occurring during breast tumorigenesis further changes in ER- α variant expression can occur during breast cancer progression. However, another study has recently found no significant differences in the relative expression of clone 4 truncated, exon 5 deleted, and exon 7 deleted ER- α mRNAs between a series of primary breast tumors and their matched concurrent lymph node metastasis (34), suggesting that altered expression of ER- α variant mRNAs likely occurs prior to the acquisition of the ability to metastasize and therefore may be a marker of future metastatic potential. This hypothesis remains to be tested.

iid. Expression of ER- α variant mRNAs and Endocrine Resistance. The hypothesis that altered forms of ER- α may be a mechanism associated with endocrine resistance has been suggested for some time. Moreover, the identification of ER- α variant mRNAs in human breast biopsy samples (23,35,36) provided good preliminary data for the hypothesis. As well preliminary functional data of the recombinant exon 5 deleted ER- α protein suggested that it possessed constitutive, hormone independent transcriptional activity which was about 15% that of the wild type ER (36). The data using a yeast expression system were also consistent with the correlation of relatively high levels of exon 5 deleted ER- α mRNA in several human breast cancer biopsy samples classified as ER- and PR+ and/or pS2+ (36, 37,38). It was also found that the exon 5 deleted ER- α mRNA was often coexpressed at relatively high levels with the wild type ER- α in many human breast cancers which were ER+ (38). Interestingly, it has been observed that transiently expressed exon 5 deleted ER- α has

an inhibitory effect on endogenously expressed wild type ER- α in MCF-7 human breast cancer cells (39), although it does not decrease the wild type activity to the same extent as hydroxytamoxifen. In contrast, in human osteosarcoma cells exon 5 deleted ER- α was shown to have little effect alone but significantly enhanced estrogen stimulated gene expression by transiently coexpressed wild type ER- α (40). The limitations of transient expression analysis was addressed by two groups who stably overexpressed the exon 5 deleted ER- α in MCF-7 human breast cancer cells (41,42). However, different phenotypes were obtained by the two groups. No effect of the recombinant exon 5 deleted ER- α on growth or estrogen/antiestrogen activity in MCF-7 cells was found in one study (41), while in the other study (42) the overexpression of recombinant exon 5 deleted ER- α in MCF-7 cells was associated with estrogen independent and antiestrogen resistant growth. The reasons for the differences between the two studies are unclear, but may be due to different MCF-7 background or additional changes which could have occurred in the transfectants in addition to transgene expression. It should be noted that the transgene in the Rea and Parker study was episomally maintained while in the study by Fuqua *et al.*, the transgene was presumably integrated into the host chromosomes in a random fashion. The results obtained using recombinant expression of a putative ER- α variant thought to have a functional role in hormonal resistance in human breast cancer, were variable suggesting possibly cell and promoter specific differences. However, irrespective of the function displayed by the exon 5 deleted ER- α under the different experimental conditions, the *in vivo* relevance of the expression levels achieved in the above experiments is likely questionable (24,39).

Several laboratories have developed cell culture models of estrogen independence and antiestrogen resistance. Variable results have been obtained when the association of altered ER- α variant mRNA expression with these phenotypes was investigated. An increased relative expression of an exon 3+4 deleted ER- α variant mRNA was found in an estrogen independent MCF-7 cell line (T5-PRF) derived by long term growth in estrogen depleted medium (43,44). However, this cell line was still sensitive to antiestrogens (43). Although one cell line that was tamoxifen resistant had differential expression of an exon 2 deleted ER- α and

an exon 5 deleted ER- α mRNA compared to the parental cell line (45), other independently derived antiestrogen resistant clones showed no major differences in the expression of ER- α variant mRNAs (46,47).

Investigation of ER- α splice variants using clinical tissue samples has also led to variable conclusions. The relative expression of the clone 4 truncated ER- α variant mRNA was significantly increased in primary breast tumors with characteristics of poor prognosis compared to tumors with good prognostic characteristics (25). As well the relative expression of clone 4 was significantly higher in PR- versus PR+ tumors, suggesting a correlation of increased truncated variant expression and markers of endocrine resistance (25). Furthermore, an increased frequency of detection of ER- α variant mRNAs deleted in exons 2-4 and 3-7 was associated with high tumor grade, but an increased detection of an exon 4 deleted ER- α variant mRNA was associated with low tumor grade (24). The presence of exon 5 deleted ER- α mRNA was found in one study to be associated with increased disease free survival (39). However, no difference in the relative expression of an exon 5 deleted ER- α variant mRNA was found between all tamoxifen resistant tumors and primary control breast tumors (37), although in the subgroup of tamoxifen resistant tumors which were ER+/pS2+, the relative expression of the exon 5 deleted ER- α was significantly greater than the control tamoxifen sensitive group.

Although increased expression of any one ER- α variant does not correlate with tamoxifen resistance of breast cancers overall, its association with and therefore possible involvement in endocrine resistance in some tumors cannot be excluded. Moreover, the presence of multiple types of ER- α variant mRNAs in any one tumor or normal tissue sample has been well documented (24,28) but no data have been published in which total ER- α splice variant expression has been analyzed in relationship to endocrine resistance and prognosis. Although mutations have been found in the ER- α gene in human breast tumors, they are rare and are not more frequent in tamoxifen resistant tumors (48).

ii. Identification of ER- α variant Proteins. An important issue remains the detection of proteins that correspond to those encoded by ER- α variant mRNAs. It is relevant therefore to understand what the structure of these proteins is. The predicted proteins of some of the most frequently detected ER- α variant transcripts are shown schematically in Figure 3. All of these variant transcripts would encode ER- α proteins missing some structural/functional domains of the wild type ER- α . While the ER- α variant transcripts encode several different types of protein, there are some common themes that emerge. A common feature of these putative proteins is the universal presence of the A/B region which is known to contain the cell and promoter specific AF-1 function. Exon 4 deleted and exon 3 + 4 deleted ER- α mRNAs are in frame and encode proteins that do not bind ligand. However, the majority of the most abundantly expressed variant transcripts i.e exon 7 deleted, an exon 4 + 7 deleted and the clone 4 truncated ER- α mRNAs encode proteins which are C-terminally truncated, and cannot bind ligand. So another common feature is the inability to bind ligand. The results obtained in which recombinant techniques were used to measure the function of individual ER- α variants *in vitro* are variable and often depend upon coexpression of the wild type receptor. While it is difficult to make general conclusions many recombinant ER- α variant proteins have been observed to modulate the activity of the wild type receptor. However, the relevance of the relative levels of expression of wild type and variant ER- α proteins that are achieved under the experimental conditions used, is unclear since limited data have been published characterizing the detection of ER- α variant proteins that are encoded by known ER- α variant mRNAs in tissues or cells *in vivo*.

From a different perspective, the prediction that the majority of ER- α variant proteins are C-terminally truncated has implications with respect to the determination of ER status as commonly assayed now. In particular, earlier detection and changes in clinical practice have resulted in smaller amounts of breast tumor tissue being available for assay, and the use of immunohistochemical methods to assess ER status becoming more common. Therefore, depending on the antibodies used, the presence of C-terminally truncated ER- α variant proteins could theoretically influence determination of ER status of the tumor sample. We have

tested this experimentally by transiently transfecting wild type ER- α and clone 4 truncated ER- α expression vectors into Cos-1 cells, and determining ER status of the cells using antibodies either to the N-terminus of the ER- α (Fig 3, 1D5, DAKO) and antibodies to the C-terminus (Fig 3, AER311, Neomarkers). Our preliminary data using different combinations of wild type ER- α and variant ER- α expression vectors transfected into Cos-1 cells indicate that the signals (expressed as H-scores which take into account the intensity of staining and the number of positively staining cells) obtained with the N-terminal and C-terminal antibodies become increasingly discrepant (N-terminal > C-terminal signal) with increasing variant expression, presumably due to increased ER- α -like proteins containing the N-terminal region but not the C-terminal region. These preliminary data suggested that increased expression of C-terminally truncated ER- α variant proteins could interfere with the immunohistochemical determination of ER status.

This possibility was investigated in human breast tumor tissues (49). A series of breast tumors was assayed for ER- α using this same set of antibodies, and the H scores from each antibody were compared for each tumor. Interestingly, the tumors fell into two distinct groups, one in which the H-scores obtained with each antibody were consistent and not significantly different from each other, and another group in which the H scores obtained with each antibody were inconsistent and significantly different from each other. Further, in all but one case the H-score was higher for the N-terminal antibody compared to the C-terminal antibody (50). As well, in preliminary experiments using a subset of the original tumor set, we have found similar results using another set of N-terminal and C-terminal ER- α antibodies. Together with the previous experimental data, one interpretation of the tumor data would be that the discrepant tumors had higher levels of C-terminally truncated ER- α -like proteins.

To address the hypothesis that the C-terminally truncated ER- α -like proteins could correspond to proteins encoded by ER- α variant transcripts, we compared expression of ER- α variant mRNAs in the consistent and inconsistent tumors. The results show a significantly higher relative expression and detection of ER- α variant mRNAs which would encode C-terminally truncated proteins in the inconsistent versus the consistent tumors (50). These

results suggest that irrespective of function the expression of significant amounts of C-terminally truncated ER- α variant proteins could interfere with the immunohistochemical determination of ER status which in turn might underlie some of the inconsistencies between ER status and clinical response to endocrine therapy. As well these data would be consistent with the hypothesis that ER- α variant mRNAs may be stably translated *in vivo*. However, such data are indirect and other mechanisms e.g. altered epitope detection, increased proteolytic activity etc, may underlie the discrepant ER- α H-scores found in some human breast tumors. More recently, data published from several independent groups support the detection of ER- α -like proteins in both cell lines and tissues *in vivo* which could correspond to those predicted to be encoded by some previously identified ER- α variant mRNAs. The presence of an exon 5 deleted ER- α protein was demonstrated immunohistochemically in some human breast tumors using a monoclonal antibody specific to the predicted unique C-terminal amino acids of the exon 5 deleted ER- α protein (39). However, although there was a correlation between immunohistochemical detection and presence or absence of exon 5 deleted ER- α mRNA determined by RT-PCR, the group was unable to detect any similar protein by Western blotting, suggesting either very low levels compared to wild type ER- α or differential stability of the variant protein relative to the wild type ER- α during the extraction procedure. In addition, an ER- α -like protein consistent with that predicted to be encoded by the exon 5 deleted ER mRNA is expressed in some BT 20 human breast cancer cell lines as determined by Western blot analysis (51). Western blotting analysis of ovarian tissue has identified both a 65 kDa wild type ER- α protein as well as a 53 kDa protein recognized by ER- α antibodies to epitopes in the N-terminus and C-terminus of the wild type protein, but not with an antibody recognizing an epitope encoded by exon 4 (52). These results correlated with the presence of both wild type and exon 4 deleted ER- α mRNAs in these tissues, and suggested that the 53 kDa protein was derived from the exon 4 deleted ER- α mRNA. More recently, a 61 kDa ER- α -like protein and a more abundant 65 kDa wild type ER- α protein were identified in MCF-7 cells (29). The 61 kDa protein is thought to be encoded by an exon 3 deleted ER- α mRNA expressed at low levels in

these cells, and its comigration both before and after dephosphorylation with the recombinant exon 3 deleted ER- α protein when expressed at higher levels after stable transgene expression in another MCF-7 clone, was thought to strongly suggest its identity with the recombinant exon 3 deleted ER- α protein.

There is accumulating evidence suggesting that variant ER- α proteins which could correspond to those predicted to be encoded by some of the ER- α variant mRNAs can be detected by conventional technologies *in vivo*.

2. Estrogen receptor- β and its Variants.

i. Identification of ER- β mRNA in Human Breast Tissues. With the discovery of a second ER, ER- β , which had similar yet distinct properties to ER- α concerning estrogen and antiestrogen action (10,11,53,54), and could directly interact with the ER- α (55,56), it became important to know if ER- β was expressed in human breast tumors and if so what role it might have in estrogen/antiestrogen action in this tissue.

We have identified the presence of ER- β mRNA both by RT-PCR (12,14) and by RNase protection assay (Figure 4) (14) in some human breast cancer biopsy samples and some human breast cancer cell lines. *In situ* hybridization analysis suggested that expression of ER- β mRNA could be detected in the breast cancer cells of a human breast cancer biopsy sample (14). Using an RT-PCR approach to analyze both ER- β and ER- α mRNA expression in a range of breast tumors (12), the following was observed: a) there was no correlation between ER- β expression and ER- α expression in breast tumors; b) in some cases both ER- β and ER- α mRNA were expressed in the same tumor; c) in those tumors where both ER mRNAs were expressed the relative expression appeared to vary widely amongst tumors. Furthermore, ER- β mRNA can be detected in normal human breast tissues by RT-PCR (13) and RNase protection assay (14). Although there are no data reporting the expression of ER- β protein(s) in human breast tissues as yet, the available information suggest that ER- β may be expressed in both normal and neoplastic human breast tissues and may have a role in these tissues.

ii.Expression of ER- β mRNA during Breast Tumorigenesis. The demonstration of ER- β mRNA expression in both human breast tumors and normal human breast tissue suggests that the well documented role of estrogen in breast tumorigenesis (1,57) may involve both receptors. Using a multiplex RT-PCR approach it has now been shown that the ER- α /ER- β ratio in a small group of ER+ human breast tumors, as assayed by ligand binding, was significantly higher than the ratio in their adjacent normal breast tissues (58). The increase in ER- α /ER- β ratio in breast tumors was primarily due to a significant upregulation of ER- α mRNA expression in all ER+ tumors in conjunction with a lower ER- β mRNA expression in the tumor compared to the normal compartment in some but not all ER+ cases. Interestingly, preliminary data suggest that the level of ER- β mRNA in breast tumors may be correlated with the degree of inflammation (unpublished data). Since *in situ* hybridization data suggest that expression of ER- β mRNA could be detected in the cancer cells of a human breast cancer biopsy sample (14) and that human lymphocytes in lymph nodes can also express ER- β mRNA (14), it is possible that the cell type contributing to the expression of ER- β mRNA may be heterogeneous depending on the tumor characteristics. Irrespective, if the RNA studies reflect the protein levels of the two ERs, the results to date provide evidence to suggest that the role of ER- α and ER- β driven pathways and/or their interaction likely changes during breast tumorigenesis.

iii. Identification of ER- β variant mRNAs in Human Breast Tissues. The presence of multiple ER- α variant mRNAs in both normal and neoplastic human breast tissues has led to the question of the expression of ER- β variant mRNAs. Several ER- β variant mRNAs have now been detected. We have identified an exon 5 + 6 deleted ER- β mRNA in human breast tumors (59). This transcript is in frame and would be expected to encode an ER- β -like protein deleted of 91 amino acids within the hormone binding domain. A human ER- β variant mRNA deleted in exon 5 was identified in MDA MB 231 human breast cancer cells and in some human breast tumor specimens (18). Although this same group was unable to detect an exon 5 deleted ER- β mRNA in normal human breast tissue, we have detected both an exon 5 deleted ER- β mRNA

and an exon 6 deleted ER- β mRNA, as well as an an exon 5 + 6 deleted ER- β mRNA in normal human breast tissue samples (13) and in some human breast tumors. The exon 5 deleted ER- β mRNA and the exon 6 deleted ER- β mRNA are out-of-frame and predicted to encode C-terminally truncated ER- β -like proteins, which would not bind ligand. More recently, several exon 8 deleted human ER- β mRNAs have been identified (17) from a human testis cDNA library and by RT-PCR from the human breast cancer cell line MDA MB 435. These variants have been named human ER- β 2-5. It should be noted that human ER- β 2 is not the equivalent of the ER- β variant mRNA with an in frame insertion of 69 nucleotides between exons 5 and 6 identified in rodent tissues (13, 60,61) and also named ER- β 2. We have been unable to detect an equivalent of the rodent ER- β 2 mRNA in any normal or neoplastic human tissue so far studied (13), suggesting species specific differences in alternative splicing of the primary ER- β transcript. Several of the human ER- β variants deleted in exon 8, specifically hER- β 2 and hER- β 5 can be detected in normal human mammary gland and in several human breast cancer cell lines (17). Interestingly, the predominant type of hER- β exon 8 deleted mRNA present varies amongst the different cell lines. We have confirmed the presence of the hER- β 2 and the hER- β 5 variant mRNAs in several normal human breast tissue samples from both reduction mammoplasties and normal tissue adjacent to breast tumors (Fig 5, unpublished data). Moreover, we have identified both hER- β 2 and the hER- β 5 variant mRNAs in several human breast tumor samples (Fig 5, unpublished data). Using a semi-quantitative RT- triple primer PCR approach (26) which simultaneously measures the relative expression of the wild type hER- β 1 and the two variants hER- β 2 and hER- β 5 mRNAs, it appears that in most but not all cases the level of the variant mRNA species exceeds that of the wild type hER- β 1 (Fig 5, unpublished data) in both normal and neoplastic human breast tissues. The known sequence of all human ER- β -like transcripts is shown schematically in Figure 6. Also shown in this figure are the proteins predicted to be encoded by these variant hER- β mRNAs. All the hER- β variant mRNAs identified to date are predicted to encode proteins which are altered in the C-terminus

in some fashion, and are unlikely to bind ligand (62). However, published data suggest that some of these variant receptors can homodimerize and bind to ERE *in vitro*, heterodimerize amongst themselves and with wild type hER- β and hER- α (17) and may potentially inhibit preferentially hER- α DNA binding transcriptional activity (62).

iv. Putative Role of ER- β and its Variants in Breast Cancer. Transient transfection studies have provided data which suggest that ER- β 1 i.e. the wild type ER- β , can only mediate an antagonist response when bound to tamoxifen-like agents, in contrast to the tamoxifen bound wild type ER- α which can mediate either an antagonist or agonist activity on a basal promoter linked to a classical ERE (53,63). This suggests the possibility that altered relative expression of the two ERs may underlie altered responses to antiestrogens, and could be a mechanism of altered responsiveness to antiestrogens in human breast cancer. As well, the activity of the estrogen bound ER- β 1 on AP-1 containing promoters is inhibitory in contrast to that of estrogen bound ER- α , which stimulates transcription (54). Furthermore antiestrogens of all types demonstrated marked transcriptional activity through ER- β 1 on promoters that contained AP1 sites (54). Interestingly, a non-ligand binding hER- β variant protein encoded by the variant hER- β 2 (also named hER- β cx) can heterodimerize with ER- β 1, but preferentially heterodimerizes with ER- α and shows a dominant negative activity only against ER- α mediated transactivation (17,62). It is possible therefore that ER- β 1 and its variants could have a direct regulatory role on ER- α activity. Since we have observed an increased ratio of ER- α /ER- β mRNA in human breast tumors compared to their adjacent matched normal tissues, which is primarily due to increased expression of ER- α mRNA in the breast tumor component (58), it is possible that this may translate into unregulated ER- α activity and unregulated growth responses mediated through ER- α which contribute to breast tumorigenesis.

However, there are several issues which have to be addressed before we can begin to develop rational pathophysiologically relevant hypotheses with respect to the role of ER- β and/or its variants in human breast tissues. Firstly, it is not known yet if ER- β and ER- α are

expressed together in the same breast cells or separately in different normal or neoplastic cell populations. Secondly, studies so far have only measured mRNA levels. No studies of protein expression with regard to ER- β -like molecules in human breast have been published to date. Therefore the pathophysiological relevance of the relative levels of ER- β and ER- α expression achieved in transient expression studies, and the resulting functional outcome are unknown. Thirdly, some *in vitro* studies have been done using N-terminally truncated ER- β 1 (64), and the functional impact of this is also unknown.

3. Expression of other Steroid Hormone Receptors and their variants in Human Breast Cancer. The observation that the PR gene showed a complex pattern of alternative splicing similar to, although not as extensive as that of ER- α , led to the further characterization of the PR variants (16,31,32). Two commonly expressed variant transcripts identified in human breast tumors and normal human breast tissue were cloned and sequenced. Variant PR mRNAs with either a precise deletion of exon 6 sequences or exon 4 sequences were identified in most breast tumors examined. PR transcripts deleted in exon 2, exons 3+6 or exons 5+6 were also found in a few breast tumors (31,32). The exon 6 deleted transcript was the most abundant and frequently expressed PR variant mRNA in the human breast tumors examined, and specific PCR primers were designed to determine the expression of this transcript relative to the wild type PR using RT-PCR analysis (27). Altered expression of ER- α variant mRNAs was observed previously between normal and neoplastic breast tissue, therefore it was of interest to determine if exon 6 deleted PR mRNA expression was altered during breast tumorigenesis. Using a similar approach to that described previously (27) the relative expression of the exon 6 deleted variant PR mRNA to the wild type PR mRNA was examined in 10 normal reduction mammoplasty samples and 17 breast tumors. The relative expression of the exon 6 deleted PR variant to the wild type PR mRNA was found to be significantly ($P < 0.01$) lower in normal breast tissues (median = 4.8%) than in breast tumors (median = 13.9%) (unpublished data).

The exon 2 deleted PR mRNA would encode a C terminally truncated PR-like protein without a DNA or a ligand binding domain (32). The exon 4 deleted PR mRNA is in frame but would encode a protein deleted in exon 4 sequences, missing a nuclear localization signal and the recombinant protein representing exon 4 deleted PR-A did not bind DNA and had little

if any effect on wild type PR-A function (32). Exon 6 deleted PR variant mRNA is out-of-frame and would encode a C-terminally truncated PR-like protein lacking the hormone binding domain, and the exon 5+6 deleted PR variant mRNA is in frame but would encode a protein deleted in exon 5 + 6 sequences of the hormone binding domain (32). Richter *et al.* have demonstrated that recombinant proteins representing the exon 6 deleted PR-A and the exon 5+6 deleted PR-A are dominant negative transcriptional inhibitors of both the wild type PR-A and PR-B (32). It is possible therefore that the presence of PR variant proteins encoded by the identified PR variant mRNAs could modify wild type PR activity and influence responses to endocrine therapies. Interestingly, small variant PR-like proteins have been identified by Western blotting in some breast tumors (32,65,66) which correspond in size to some of the proteins predicted to be encoded by some of the exon deleted PR mRNAs. However, some data suggest that the presence and abundance of PR variant mRNAs may not correlate with the detection of these smaller sized PR immunoreactive species in human breast tumors (66).

The measurement of PR is an important tool in clinical decision making with respect to prognosis and treatment of human breast cancer. Furthermore, the level of PR expression provides important clinical information (67). As the use of enzyme-linked immunosorbent assays (ELISA) and immunohistochemical assays for PR detection increases, it is likely that variant PR expression will interfere with these assays, whatever their function. PR antibodies (AB-52 antibody) used in such assays detect epitopes in the N-terminal region of the wild type molecule shared by truncated PR-like molecules. If all or any of the deleted PR variant mRNAs so far identified are translated into stable proteins, they will be co-detected with the wild type PR in such assays. Presence of PR variants might also be a factor contributing to discrepancies between biochemical measurement and immunological detection of PR. Indeed the potential for ER- α variant expression to interfere with the immunohistochemical assessment of ER status has been documented (49,50,68).

4. Conclusions and Controversies.

The multifaceted nature of the ER is suggested by the expression of ER- α mRNA, ER- β mRNA and their variant mRNAs in both normal and neoplastic human breast tissues (Figure 7). There is a large body of molecular data which support at least the potential for this multifaceted nature of the ER and therefore estrogen/antiestrogen signalling in both normal

and neoplastic human breast tissues. Alterations in the relative expression of several ER-like mRNAs have been shown to occur during breast tumorigenesis and the relative frequency of detection and relative expression of individual ER-like mRNAs can be correlated with different prognostic characteristics in breast cancer. This in turn suggests a possible role in breast tumorigenesis and possibly hormonal progression in breast cancer. However, there are still major gaps that need to be addressed before we have a clear idea of the pathophysiological and functional relevance of the experimental results so far in hand. Unequivocal data are required to support the *in vivo* detection of variant ER- α , variant ER- β and wild type ER- β proteins which correspond to the variant ER- α , variant ER- β and wild type ER- β mRNA species, respectively. There is a need to experimentally determine putative function using expression levels which reflect pathophysiological levels of expression. There is a need to know if the two wild type ER receptors and/or their variants are co-expressed in the same cells within the heterogeneous normal and neoplastic breast tissues. Further, given the detection of multiple forms of variant ER-like species in any one breast tissue sample the limitations in interpreting data from experimental systems in which only one variant species is considered in the presence or absence of wild type protein needs to be understood.

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Figure Legends.

Figure 1.

Top panel. Schematic representation of wild type ER- α (WT-ER) cDNA and primers allowing co-amplification of most of the described exon-deleted ER- α variants: ER- α cDNA contains 8 different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another trans-activating function (AF-2). 1/8U and 1/8L primers allow amplification of 1381 bp fragment corresponding to wild type ER- α mRNA. Co-amplification of all possible exon-deleted or inserted variants that contain exon 1 and 8 sequences can occur. Amplification of the previously described ER- α variant mRNAs deleted in exon 3 (D3-ER), exon 4 (D4-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER), exons 4 and exon 7 (D4/7-ER), would generate 1264 bp, 1045 bp, 1197 bp, 928 bp, 1073 bp and 861 bp fragments, respectively.

Bottom panel. Co-amplification of wild type ER- α and deleted variant mRNAs in breast tumor samples: Total RNA extracted from ER positive (+) and ER negative (-) breast tumors was reverse transcribed and PCR amplified as described (24) using 1/8 U and 1/8L primers. Radioactive PCR products were separated on a 3.5% acrylamide gel and visualized by autoradiography. Bands reproducibly obtained within the set of tumors studied and that migrated at 1381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp and 580 bp were identified as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3 and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2, 3 and 4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. PCR products indicated by dashes (-), barely detectable within the tumor population, i.e present in less than or equal to 3 particular tumors, have not yet been identified. M: Molecular weight marker (phi174, Gibco BRL, Grand Island, NY). Adapted from (24).

Figure 2.

Linear regression analysis of the relationship between the clone 4 truncated ER- α mRNA and the wild type ER- α mRNA in the various groups. Closed circles represent the "good" prognosis/ER+PgR+ group; open circles represent the "good" prognosis/ER+PgR- group;

closed squares represent the "poor" prognosis/ ER+PgR+ group; open squares represent the "poor" prognosis /ER+PgR- group. Good vs Poor, $p = 0.0004$; PgR- vs PgR+. $P = 0.011$. Reproduced from (25).

Figure 3.

Schematic representation of the ER- α variant proteins predicted to be encoded by ER- α variant mRNAs. Identical sequence is depicted by numbered exons. U = amino acid sequence unrelated to wild type human estrogen receptor- α amino acid sequence. U sequences are unique to any particular variant. The position of N- and C-terminal epitopes recognized by 1D5 and AER311 antibodies, respectively, are indicated.

Figure 4.

Detection of ER β mRNA in human breast tumors by RNase protection assay. A. Schematic representation of hER β mRNA showing various exon sequences and identifying the riboprobe position and size of the expected protected fragment (259 bp). Total RNA was isolated from 7 breast tumor samples and 25 μ g were used in an RNase protection assay as previously described (21). Ovarian RNA (ovary) was used as a positive control.

Figure 5.

RT-triple primer PCR analysis (26) of the relative expression of human estrogen receptor- β 1 (ER- β 1), human estrogen receptor- β 5 (ER- β 5) and human estrogen receptor- β 2 (ER- β 2) mRNAs in normal (N) and breast tumor (T) tissue samples.

Figure 6.

Human ER- β isoforms. All hER- β isoforms are aligned. White boxes indicate identity of amino acid between sequences. Amino acid positions of the different structural domains are indicated for the hER- β 1 short (14) that contains 8 extra N-terminal amino acids compared to the first hER- β described (10). hER- β 1 long (Genbank AF051427) contains 45 additional N-terminal amino acids. hER- β 1 Δ 5 (13,18), hER- β 1 Δ 6 (13), hER- β 2 (Genbank AF051428, AB006589cx), hER- β 3 (Genbank AF060555), hER- β 4 (Genbank AF061054), hER- β 5 (Genbank AF061055) are truncated and contain different C-terminal amino acids (black boxes). hER- β Δ 5-6 (13)(Genbank AF074599) is missing 91 amino acids within the LBD/AF2

domain. For each receptor, the length (aa) and the calculated molecular mass (kDa) when known or corresponding to the short (S) or the long (L) forms of the putative proteins are given. Broken boxes and question marks indicate that flanking amino acid sequences are unknown.

Figure 7.

Schematic representation of the known and unknown (?) multiple facets of the estrogen receptor (R).

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Figure 1

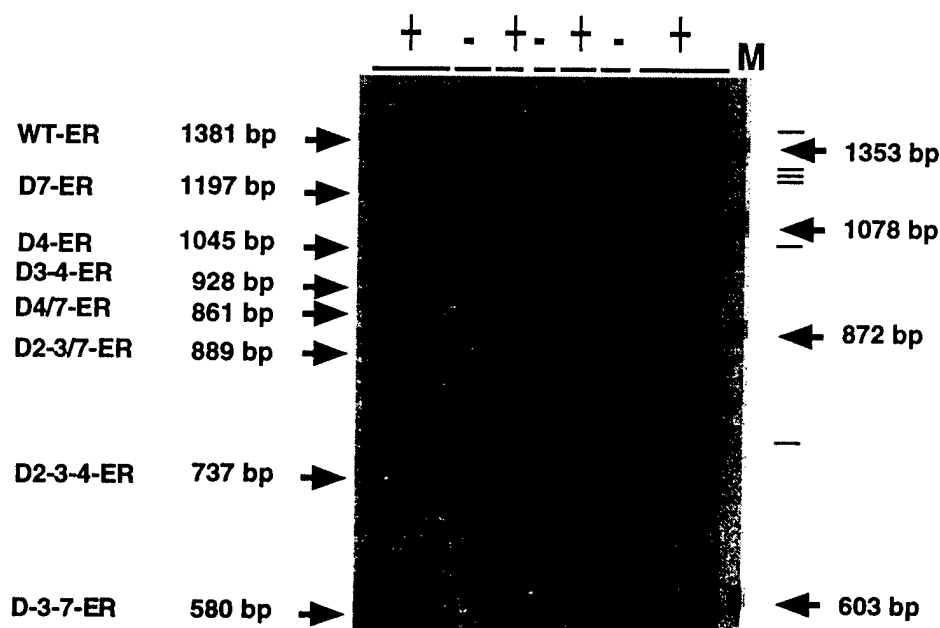
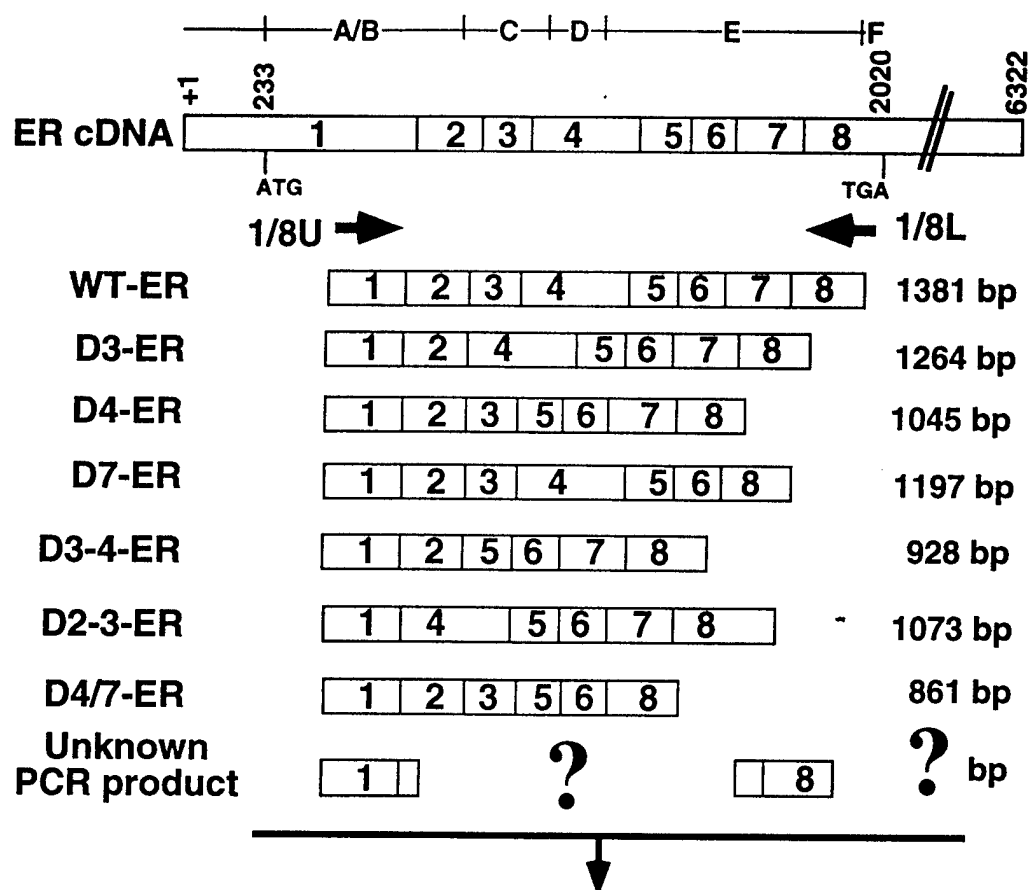


FIGURE 2.

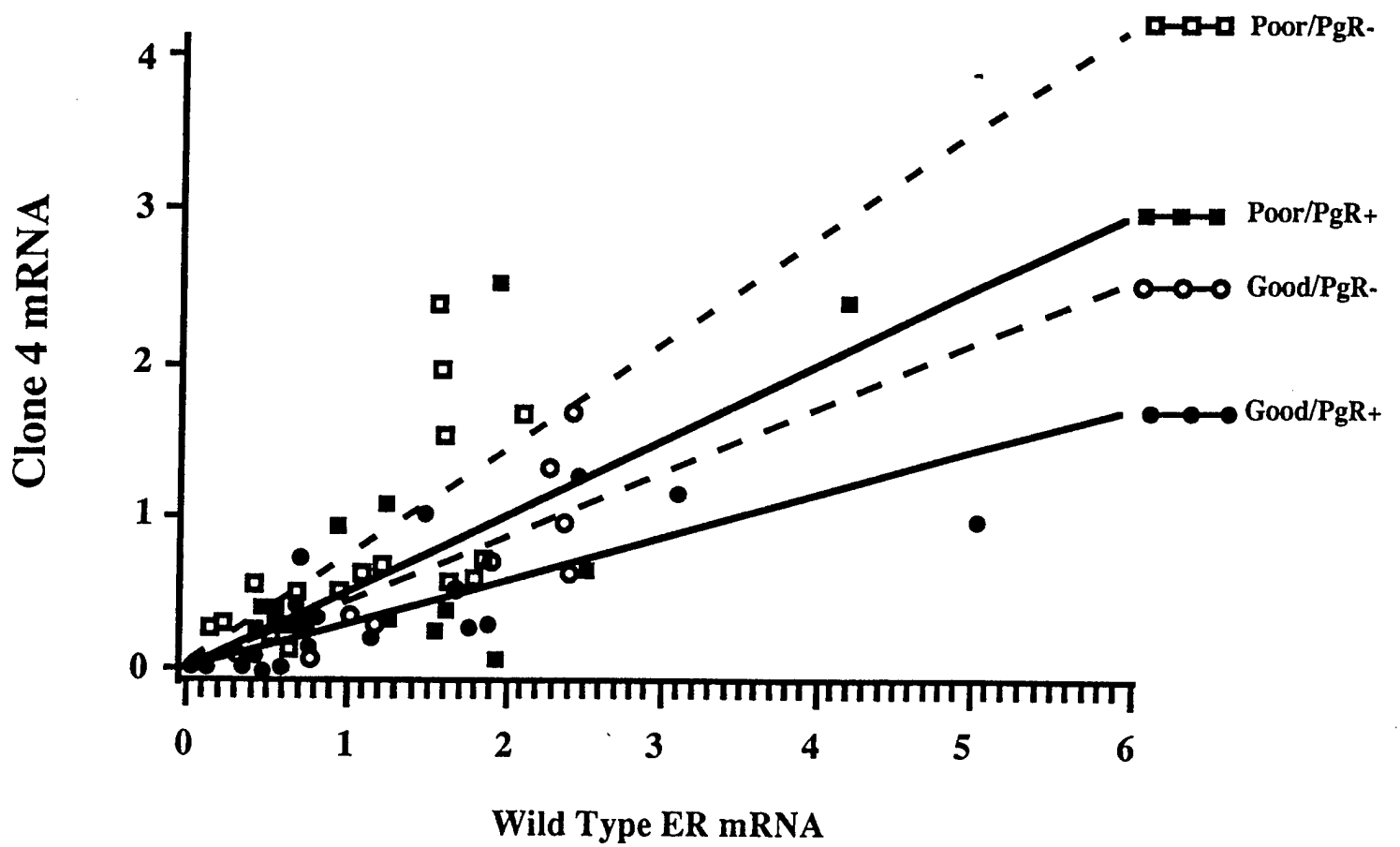
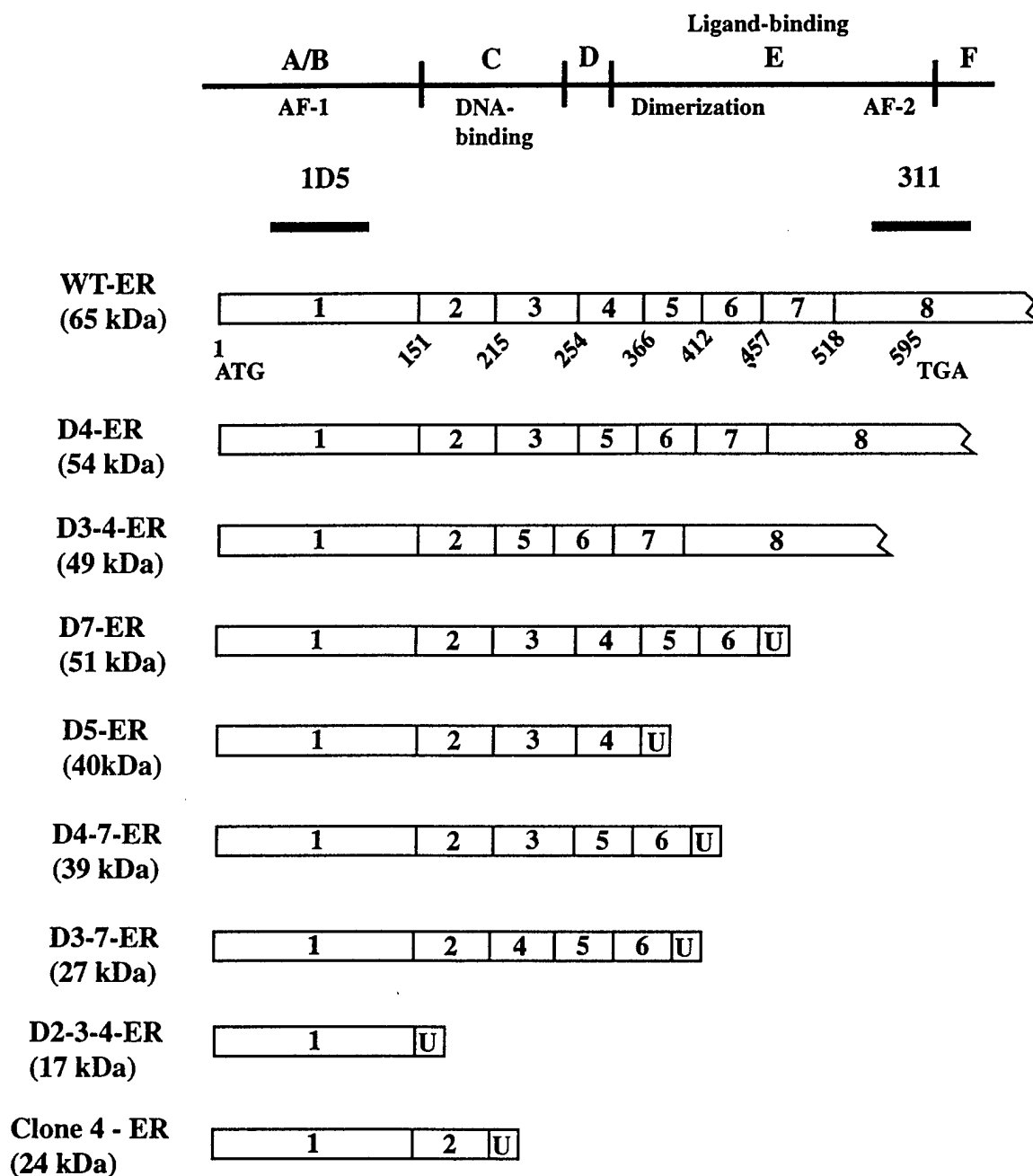


Figure 3



A.

ER- β
mRNA



Riboprobe - 259 bp

B.

Breast Tumors

1 2 3 4 5 6 7

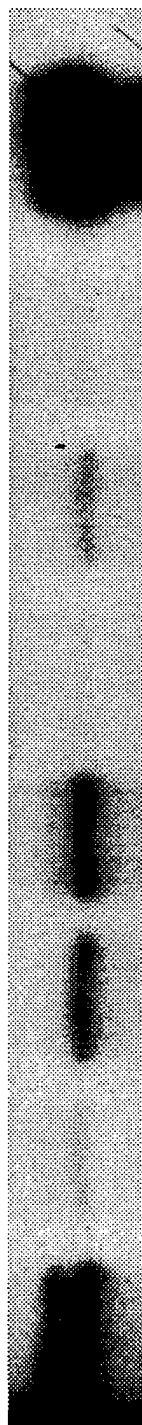
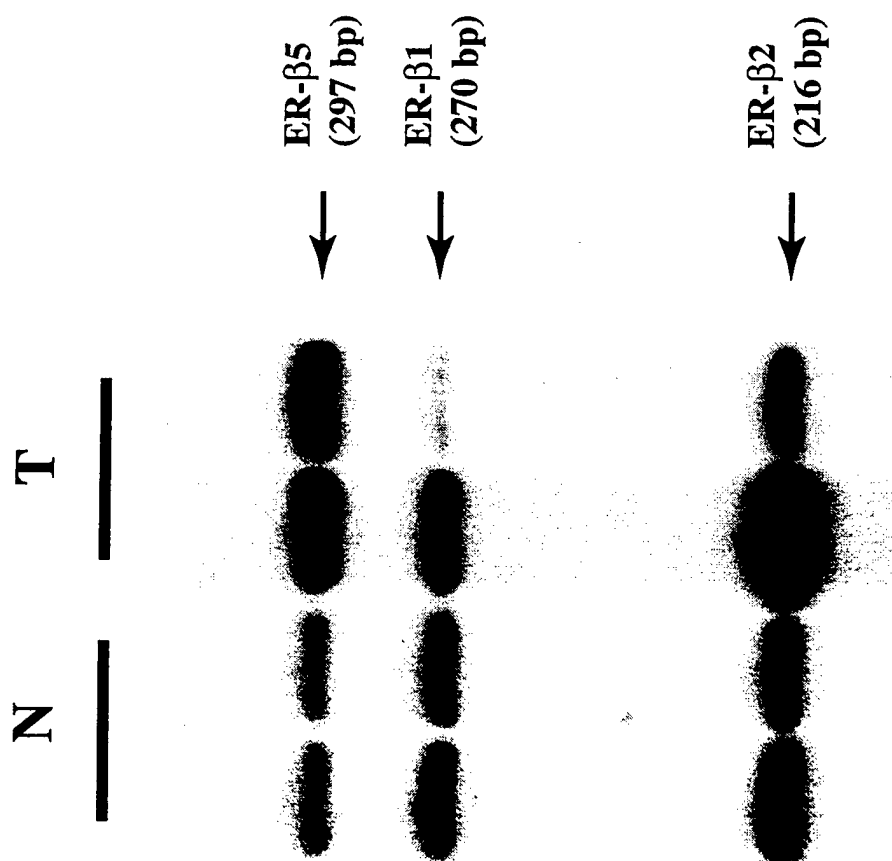


Figure 4



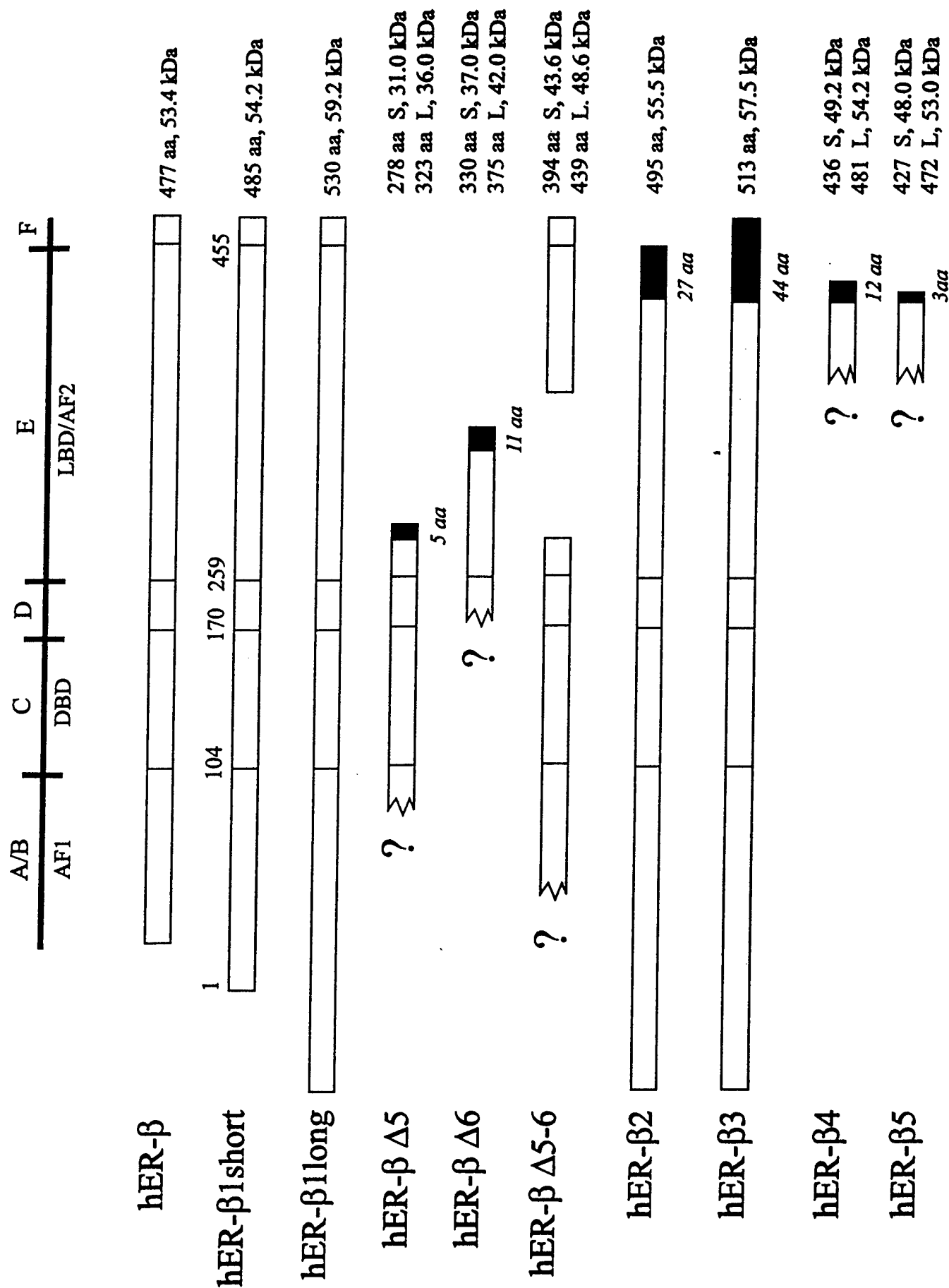
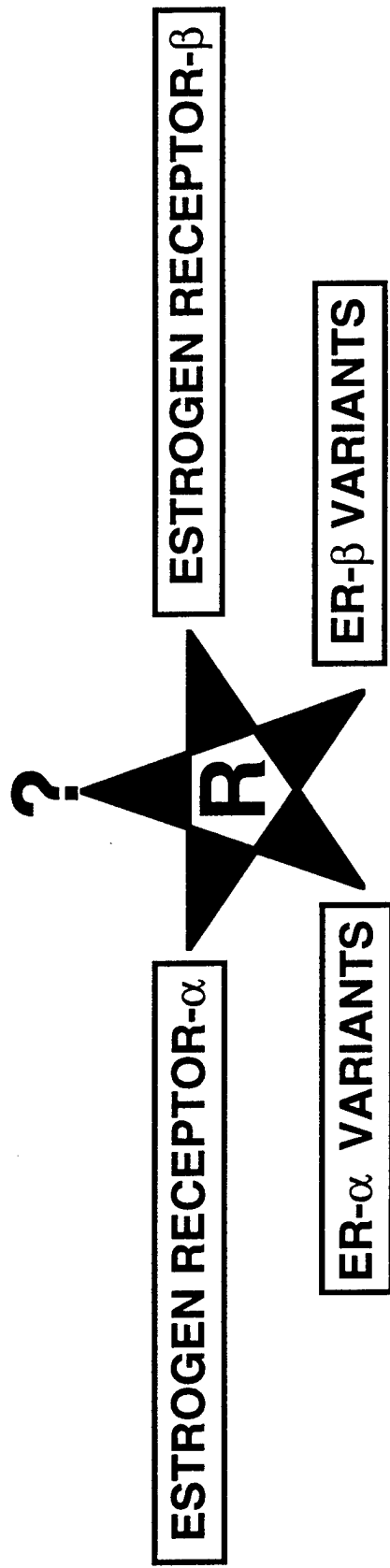


Figure 7.



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APPENDIX 25

Lu B, Dotzlaw H, **Leygue E**, Watson PH, and Murphy LC

Estrogen receptor- β (ER- β) variants in human in breast tissues.

Breast Cancer Research: Reasons for Hope. Canadian Breast Cancer Research
Initiative National Scientific Conference.
Toronto, Ontario, Abstract number to be announced, 1999.

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ESTROGEN RECEPTOR- β (ER β) VARIANTS IN BREAST TISSUES.

Biao Lu MD¹, Helmut Dotzlaw¹, Etienne Leygue PhD¹, Peter Watson MD², Leigh C Murphy PhD¹. Department of Biochemistry and Molecular Biology; University of Manitoba, Winnipeg, Manitoba¹. Department of Pathology; University of Manitoba, Winnipeg, Manitoba².

Recently we identified the expression of ER β mRNA in human breast tissues. We have previously identified several variant ER α mRNAs in human breast tissues, and now investigate the expression of similar ER β mRNA variants in human tissues. Mouse models are useful to investigate putative function in the whole organism, so we have also studied the expression of ER β mRNA variants in mouse tissues. ER β mRNA splice variants were identified in human breast tumors and in normal human and mouse ovarian, uterine and mammary tissues. In both species mRNAs deleted in exons 5 or 6, or 5+6 were characterized. In mouse tissues an abundant ER β mRNA containing 54 nucleotides inserted in-frame between exons 5 and 6 was identified and is referred to as mER β 2 compared to wild type mER β 1. The predicted mER β 2 protein contains 18 amino acids inserted in the ligand binding domain of mER β 1. Recombinantly expressed mER β 2 did not bind ligand but bound with similar affinity and DNA bending activity as mER β 1 to EREs *in vitro* and *in vivo*. Transcriptional activity was assessed using transient transfection into NIH 3T3 cells, with classical and non-classical estrogen regulated reporter genes i.e. ERE-tk-CAT, AP1-CAT and TGF- β 3-CAT. mER β 2 differentially regulated these promoters. The relative level of expression of mER β 2 and mER β 1 mRNA in various mouse tissues indicated that mER β 2 mRNA was predominant in some but not all tissues. The data suggest that mER β 2 may have tissue and promoter specific modulatory effects which could contribute to tissue specific estrogen action. But, no equivalent of the mouse inserted transcript was detected in any human tissues analyzed, suggesting species specific alternative splicing and possibly function. However, different variant human ER β mRNAs were recently described by others in several human tissues. Among them, an ER β 2 variant, deleted in exon 8 of the hER β 1 (not equivalent to mER β 2) was shown to heterodimerize with hER β 1 and hER α and inhibit ER α activity. This raised the possibility that such variants may interfere with both ER α and ER β function and influence mechanisms underlying breast tumorigenesis and breast cancer progression. We measured the expression of these hER β variant mRNAs in human breast tissues using a novel technique called triple primer polymerase chain reaction (TP-PCR). Our data suggest that changes in the relative expression of ER β 1 and its variants occurs during breast tumorigenesis and tumor progression.

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APPENDIX 26

Dotzlaw, **Leygue E**, Huang A, Snell L, Watson PH, and Murphy LC

Estrogen receptor (ER) variants in human breast cancer.

Breast Cancer Research: Reasons for Hope. Canadian Breast. Cancer
Research Initiative National Scientific Conference.
Toronto, Ontario, Abstract number to be announced, 1999.

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ESTROGEN RECEPTOR (ER) VARIANTS IN HUMAN BREAST CANCER.

Helmut Dotzlaw¹, Etienne Leygue PhD¹, Aihua Huang MSc², Linda Snell², Peter Watson MD², Leigh C Murphy PhD¹. Department of Biochemistry and Molecular Biology; University of Manitoba, Winnipeg, Manitoba¹. Department of Pathology; University of Manitoba, Winnipeg, Manitoba².

The progression of breast cancer from hormone dependence to independence is a clinical problem which limits the effectiveness of endocrine therapies. These therapies aim to inhibit the growth promoting effect of estrogen on breast cancer cells. However, despite the continued expression of ER a large number of breast cancers develop mechanisms short circuiting their requirement for estrogen, resulting in the development of estrogen independence and eventually resistance to endocrine therapies. The studies presented address a possible mechanism for this progression by addressing the hypothesis that the altered expression of ER variant proteins in human breast cancer is associated with the progression from hormone dependence to independence and the development of endocrine resistance. Using recombinant DNA technology we have identified and characterized several variant ER α mRNAs in human breast cancers as well as in normal human breast tissues. The relative expression of some but not all variant ER α mRNAs was significantly increased in breast tumor tissue compared to normal breast tissue. The data suggest that although the mechanism(s) for generating variant ER α mRNA exists in normal breast tissue it may be deregulated during breast tumorigenesis. Furthermore, altered expression of some variant ER α mRNAs was found to be associated with known prognostic and treatment response markers in breast cancer. We have also correlated increased expression of variant ER α mRNAs with increased immunohistochemical detection of C-terminally truncated ER-like proteins, which is consistent with the detection of ER α variant proteins corresponding to ER α variant mRNAs *in vivo* in human breast tumors. We have recently reported the detection of ER β mRNA in both normal and neoplastic human breast tissues. The relative expression of ER α /ER β mRNAs changes between normal breast tissues and their concurrent matched ER+ breast tumors suggesting that altered expression of ER α and ER β may be functionally involved in breast tumorigenesis. As well the level of ER β mRNA varies between breast tumors and is inversely correlated with progesterone receptor expression, a marker of endocrine therapy sensitivity. In conclusion our data suggest that altered expression of ER variants and/or isoforms occurs during breast tumorigenesis and breast cancer progression, and supports the hypothesis that altered expression of these isoforms may be a mechanism associated with breast cancer progression and development of endocrine resistance.

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APPENDIX 27

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Expression of estrogen receptor beta 1, beta 2 and beta 5 mRNAs
in human breast tissue.

81th Annual Meeting of the Endocrine Society. San Diego, California,
Abstract number P3-265, 1999.

72 h later (n=4 per group). After collecting the uteri, a section of one uterine horn from each animal was cryopreserved for *in situ* hybridization. The remaining uterine tissue was homogenized for total cellular RNA purification and northern blot analysis using ³²P-labeled cDNAs encoding SPA (switch protein for antagonist, 0.8kb), RIP140 (receptor interacting protein, 7.2kb), SRC-1 (steroid receptor coactivator, 3kb), GRIP (glucocorticoid receptor interacting protein, 1kb), p300 (9kb), and RAC3 (receptor associated coactivator, 2.3kb). ³⁵S-labeled riboprobes corresponding to SPA, SRC-1, RIP140, p300, and GRIP were used for *in situ* hybridization. Although expression of the coactivators was detectable by northern analysis, no change in coactivator transcript levels was observed at any time points after hormone or antihormone administration. Interestingly, among the coactivators, SPA mRNA showed a distinct high expression level in the uterus. *In situ* hybridization was used to determine the cell specific pattern of coactivator expression. The results revealed the appearance of silver grains corresponding to the coactivators in all uterine tissue compartments (epithelium, stroma and myometrium); however, mRNA expression of the indicated coactivators appeared elevated in luminal and glandular epithelial cells. Also, after estrogen or progesterone administration, there was no distinct change in silver grain pattern or intensity, which supports our northern analysis data. In summary, the coactivators SPA, SRC-1, p300, RIP140, GRIP, and RAC3 do not appear to be induced by female sex steroids in rat uterus; however expression of the coactivators appeared more distinct in the uterine epithelium compared to stroma and myometrium (supported by NIH 463068 to K. P. N.).

P3-264

FUNCTIONAL ROLES OF UTERINE ESTROGEN RECEPTORS (ER): ACTIVATION OF IGF-1 AND INHIBITION OF IGFBP-2 GENE TRANSCRIPTION BY ER- α , BUT NOT ER- β , IN HUMAN ENDOMETRIAL ADENOCARCINOMA CELLS (Hec-1-A).

[Kwak,* RCM Simmen, F A Simmen. Interdisciplinary Concentration in Animal Molecular and Cell Biology, University of Florida, Gainesville, FL

The local growth-promoting action of the uterine insulin-like growth factor (IGF) system is well documented. Previously, we showed that expression of the IGF-1 gene in porcine endometrium is significantly higher during early than later pregnancy. Conversely, expression of the IGFBP-2 gene in endometrium is greatest during later pregnancy. Subcutaneous administration of estrogen (E2) to immature or ovariectomized mature pigs increases uterine IGF-1 gene expression, but the molecular mechanisms underlying uterine IGF-1 and IGFBP-2 gene regulation by E2 are poorly understood. In this study, we examined the transcriptional activities of porcine IGF-1 and IGFBP-2 gene promoter-reporter constructs after co-transfection with human ER- α and rat ER- β expression vectors. The -1998 fragment [initiated at position -1998, and extending to -21 nucleotide (nt); +1=translation initiation codon for the pre-propeptide encoded by exon I] of IGF-1 and the -1397 fragment [initiated at position -1397, and extending to +73 nt of exon I] of IGFBP-2 were linked to a luciferase reporter gene in the vector pGL3-basic. Both constructs were transiently transfected into Hec-1-A (human endometrial adenocarcinoma) cells, and the cells incubated in complete medium (McCoy's 5A) for 2 days prior to assay for luciferase activity. For E2 treatment, charcoal-stripped serum and phenol red-free (CSPR-) Opti-MEM medium was used. Co-transfection of ER- α and IGF-1 constructs in the absence of E2 showed a significant increase (5-8 times higher than control) of IGF-1 transcriptional activity. Although E2 had only a minimal effect on IGF-1 transcriptional activity in the absence of co-transfected ER- α , E2 treatment with ER- α co-transfection yielded a 50% increase over ER- α alone in (CSPR-) Opti-MEM. ER- α activation of IGF-1 promoter activity was responsive to increasing ER- α concentration but not to E2 concentration. Co-transfection of ER- α and the IGFBP-2 promoter construct in the absence of hormone showed significant decreases (6-9 times lower than controls) of IGFBP-2 transcriptional activity. However, co-transfection of ER- β with IGF-1 or IGFBP-2 constructs showed moderate inhibition (2-4 fold decrease). Results suggest differential regulation of uterine IGF-1 and IGFBP-2 genes by the two ER isoforms. (Supported by NIH HD 21961)

P3-265

EXPRESSION OF ESTROGEN RECEPTOR BETA 1, BETA 2 AND BETA 5 mRNAs IN HUMAN BREAST TISSUE.

Etienne Levgue,*¹ Helmut Dotzlaw,² Peter H Watson,² Leigh C Murphy.¹ ¹Biochemistry, University of Manitoba, Winnipeg, MB, Canada, ²Pathology, University of Manitoba, Winnipeg, MB, Canada

Several variant forms of estrogen receptor (ER) beta 1 mRNA have recently been identified in several human tissues including breast. Among them, an ER- β variant, deleted of regions encoded by ER- β exon 8 sequences, has been shown to heterodimerize with both ER- β 1 and ER- α and to inhibit ER- α DNA binding capability. The ability of ER- β variants to potentially interfere with ER- α and ER- β 1 signaling pathways raised the question of their possible involvement in mechanisms underlying breast tumorigenesis and tumor progression. We have developed a triple primer polymerase chain reaction (TP-PCR) assay to evaluate the relative expression of ER- β 1, ER- β 2, ER- β 4 and ER- β 5 mRNAs. TP-PCR, based on the co-amplification of ER- β 1, ER- β 2, ER- β 4 and ER- β 5 cDNAs, was used to investigate the relative expressions of the corresponding mRNAs in breast cancer lines and in 53 independent breast tumors. The expression of ER- β 2 and ER- β 5 mRNAs was higher than that of ER- β 1 mRNA in both cancer cell lines and breast tumors (Wilcoxon sign rank test, n = 53, p < 0.005). In breast tumors, an increase in the ratio of ER- β 2/ER- β 1 and ER- β 5/ER- β 1 mRNA expression was observed, that positively correlated with the level of tumor inflammation. (n = 52, Spearman r = 0.34, p = 0.01) and

tumor grade (n = 47; Spearman r = 0.29, p = 0.04), respectively. A trend towards an increase of these ratios was also found in tumors, as compared to the normal adjacent breast tissue available for 13 cases. Our data suggest that changes in the relative expression of ER- β 1, ER- β 2, and ER- β 5 mRNAs occur during breast tumorigenesis and tumor progression.

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17 β -ESTRADIOL DOWN-REGULATES INDUCIBLE NITRIC OXIDE SYNTHASE IN ENDOTHELIAL CELLS THROUGH A NON-CLASSICAL MECHANISM.

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Substantial data suggests that 17 β -estradiol exerts its cardioprotective effects in part by increases in the endothelial form of nitric oxide synthase (eNOS) in endothelial cells, thereby leading to increased production of the potent vasodilator, nitric oxide (NO). In addition to eNOS, endothelial cells can express an inducible isoform of nitric oxide synthase, iNOS. Paradoxically, the induction of iNOS and subsequent excess production of NO is believed to play a role in the pathogenesis of septic shock, atherosclerosis, and vascular inflammatory disorders. The effect of estradiol on the induction of iNOS, however, is not known. We measured the effect of physiological concentrations of 17- β estradiol on the induction of iNOS protein and NO production in cultured rat aortic endothelial cells (RAECs). Cells were cultured in DMEM containing 10% FBS, 2 mM glutamine and 25 μ g/100 ml of gentamicin at 37°C with 5% CO₂ and 95% humidity. Before experiments, cells were made quiescent in FBS-free DMEM for 12 h. Cell cultures were then pre-incubated with vehicle or estradiol (0.001 - 10 nM) for 1h, followed by incubation with interleukin-1 β (IL-1 β , 10 ng/ml) with or without estradiol for 24 h. iNOS was measured by immunoblotting using a monoclonal antibody (1:1500, Transduction Laboratory, Lexington, KY). NO was determined by measuring nitrite accumulation in media using the Griess reaction. Estradiol at all doses reduced the induction of iNOS by IL-1 β ; reductions were significant at estradiol concentrations of 0.1 and 10 nM (p<0.05). The reduction was dose-dependent; the greatest reduction, approximately 30%, was observed at 10 nM estradiol. The same concentrations of estradiol also significantly inhibited nitrite accumulation (p<0.001). In this case, however, the reduction was not dose-dependent; all concentrations reduced nitrite accumulation by 25 - 30%. Surprisingly, the estrogen receptor antagonist ICI 182,780 (10 nM) had no effect on the ability of estradiol (0.1 nM) to decrease iNOS protein levels or reduce nitrite accumulation. ICI 182,780 by itself had no effect on iNOS protein or nitrite accumulation. These results suggest estradiol may protect against excessive NO production by iNOS in endothelial cells. Furthermore, the inhibitory effects of estradiol on iNOS and NO production appear to be mediated through a nonclassical mechanism. Supported by the Alumnus Vascular Biology Program, the Karmanos Cancer Institute and VA Research Funds.

P3-267

17 β -ESTRADIOL INHIBITS THE PROLIFERATION INDUCED BY INSULIN IN FEMALE RAT VASCULAR SMOOTH MUSCLE CELLS.

N. Itabashi,* N. Fujita, Y. Tuboi, S. Fukuda, K. Okada, T. Saito. Department of Medicine, Jichi Medical School, Tochigi, Japan

The previous studies demonstrated that insulin activates the proliferation of vascular smooth muscle cells (VSMC). In contrast, 17 β -estradiol (E2) inhibits the proliferation of female VSMC. Therefore, we investigated the cellular mechanisms of E2 to inhibit the proliferation induced by insulin in cultured female rat VSMC. The cellular mechanisms were revealed by examining ³H-thymidine incorporations implicated in DNA synthesis and the expression of cdk2 protein to regulate G1/S transition. E2 (0.1 μ M) reduced the ³H-thymidine incorporations induced by 5% dextran-coated charcoal-stripped-FCS (DCC-FCS) with a peak at 24h (5%DCC-FCS, 5%DCC-FCS+0.1nM E2 : 0h; 2069.9 vs 2069.9, 3h ; 1724.5 vs 2503.2, 6h; 3396.8 vs 2766.5, 12h; 11179.3vs 8893.6*, 24h; 25548.5vs 20313.6*, 48h; 11639.3 vs 9549.3* CPM/mg protein, n=6, *p<0.01) without any effect on basal levels. Insulin (0.1 μ M) increased the ³H-thymidine incorporations with a peak at 24h (basal; 2069.9, 6h; 2092.3, 12h; 3345.9*, 24h; 5750.2*, 48h; 5310.4* CPM/mg protein, n=6, *p<0.01 vs basal). E2 (0.1 μ M) reduced the ³H-thymidine incorporations induced by insulin (0.1 μ M) at 24h (Control: 1645.9, Insulin: 3397.0, Insulin+E2: 2162.8 CPM/mg protein, n=6, p<0.01). We examined the effects of E2 on the expression of cdk2 protein. Insulin and 5%DCC-FCS activated the expression of cdk2 in female VSMC. Western blotting showed that the treatment of E2 reduces an increase in the expression of cdk2 protein induced by 5%DCC-FCS and insulin. We concluded that 17 β -estradiol prevents the G1/S transition.

APPENDIX 28

Dotzlaw H, **Leygue E**, Watson PH, and Murphy LC

Relationship of estrogen receptor beta (ER- β) mRNA expression to markers of endocrine therapy sensitivity in human breast cancer.

90th Annual Meeting, American Association for Cancer research (AACR).
Philadelphia, Pennsylvania, Abstract 2516, 1999.

paraffin embedded tumors by FCM. Fourteen of the 20 samples (70%) analyzed were positive for ER expression by both cytosolic EIA and FCM analysis. 7/11 specimens were ER positive by FCM and immunohistochemical (IHC) analysis while 3/11 specimen were FCM negative but IHC positive. In PGR analysis, 11/20 (55%) samples were positive by both the assays. 10/11 samples were positive by FCM and IHC while 1/11 was FCM positive but IHC negative for PGR expression. Observation in the present study show that improved FCM procedures can be used for multiparametric analysis of hormone receptor expression in breast tumors.

#2514 Estrogen-induced iron accumulation prior to carcinogenesis. J. Shawn Jones, Mary L. Thomas, Nancy W. Alcock, Joachim G. Liehr. *Dept. of Pharmacology, University of Texas Medical Branch at Galveston, Galveston, TX 77555.*

Iron is an essential nutrient of cells and is utilized in metabolic processes and in cell proliferation, whereas iron overload has been associated with increased risk for cancer. We have previously shown that hamsters fed a Teklad diet supplemented with iron (87% over normal rodent chow) doubled the incidence of estrogen-induced kidney tumors (Wyllie and Liehr, 1998). Our hypothesis is that estrogen stimulates accumulation of iron in tissues where this hormone induces tumors. The excess iron may cause free radical-mediated DNA and protein damage and contribute to tumor formation. We are investigating this hypothesis by studying the accumulation and/or redistribution of iron in response to estrogen in nine different tissues of the Syrian hamster, an animal model of estrogen-induced kidney tumorigenesis. Hamsters were treated by subcutaneous implantation of a 25 mg estradiol pellet for 3 or 7 days. The tissues were processed and analyzed for total iron content by atomic absorption spectrophotometry. Estradiol induced an accumulation of iron in kidney cortex over controls by 38% and 52% at 3 and 7 days, respectively, and decreased levels in the lung by 31% and 53% at 3 and 7 days, respectively. It also induced a transient increase in spleen iron of 36% over controls at 3 days, followed by a decrease of 16% below controls at 7 days. This pattern suggests an estrogen-induced redistribution of iron from lung and spleen to kidney cortex, the target tissue for tumor formation. If this pattern is observed in target tissues of other species, it may indicate a role for iron in hormone-associated cancers. Supported by a grant from NIH, National Cancer Institute (#CA 74971).

#2515 16-Hydroxylation of steroidal estrogens by rodent and human microsomes. Somasunderam, Anoma, Zhu, Bao T., Hammond, Dianne K., Hanania, Taleen, and Liehr, Joachim G. *University of Texas Medical Branch at Galveston, Galveston, TX 77555.*

Estradiol (E_2) has been postulated to be metabolized to 16 α -hydroxyestrone (16 α OH- E_1) and estriol (E_3) by initial formation of estrone (E_1) and its subsequent 16-hydroxylation. 16 α OH- E_1 has been postulated to play a role in the development of breast cancer. As part of our examination of this hypothesis, we have established validated product isolation assays using gas chromatography and determined the enzyme activities for the formation of these metabolites in both human and rodent liver microsomes. The rate of formation of E_3 and 16 α OH- E_1 from 10 μ M E_2 by rat liver microsomes were 295 and 94 pmol/mg protein/min respectively. The rates of metabolic conversion of E_1 to 16 α OH- E_1 by microsomes was 39 pmol/mg protein/min. In order to determine whether E_3 was formed by direct 16-hydroxylation of E_2 or by intermediate formation of E_1 and 16 α OH- E_1 , 17 α -deuterated E_2 (synthesized by reduction of estrone with NaBD $_4$) was incubated with rat liver microsomes. The E_3 metabolite formed retained the deuterium label at the 17 α position (>95%). The formation of this metabolite via E_1 and 16 α OH- E_1 was expected to result in loss of the 17 α -deuterium label. A similar experiment with human liver microsomes produced mainly catechol metabolites and E_1 , without detectable 16 α -hydroxylated metabolites. Our data demonstrate that E_3 is preferentially formed by direct 16 α -hydroxylation and only to a minor extent via estrone as previously postulated. In these experiments with liver microsomes, the 16 α -hydroxylation is a minor metabolic pathway with rates approximately 10–20% and 20–30% of those of 2- and 4-hydroxylation, respectively. Supported by the National Cancer Institute, NIH (CA 63129).

#2516 Relationship of estrogen receptor (ER)- β mRNA expression to markers of endocrine therapy sensitivity in human breast cancer. Dotzlaw, H., Leygue, E., Troup, S., Watson, P.H., Murphy, L.C. *Dept. of Biochemistry & Molecular Biology; *Dept. of Pathology, University of Manitoba, Winnipeg, Canada. R3E 0W3.*

The level of ER- β mRNA in breast tumors, determined by RT-PCR and normalized to GAPDH mRNA levels, was compared to ER and PR status determined by ligand binding assays as surrogate markers of sensitivity to endocrine therapy. The level of ER- β mRNA was significantly lower in PR+ tumors compared to PR- tumors ($p = 0.036$) and no association with ER status was found. Subgroup analysis showed that ER- β expression in ER+/PR+ breast tumors was significantly less than in ER+/PR- ($p = 0.009$), ER-/PR+ ($p = 0.029$) and ER-/PR- ($p = 0.023$) groups. The relationship of ER- β mRNA levels with PR status in breast tumors suggested that ER- β expression may be regulated by progestins. This was investigated using the PR+ T-47D cell line in culture. Progestins specifically decreased the steady state levels of ER- β mRNA in a time and dose dependent fashion in T-47D cells. The data suggest that expression of ER- β in human breast tumors may be a marker of endocrine therapy responsiveness.

#2517 Effect of PPAR γ and RAR specific ligands on breast cancer cell lines. Elstner, E., Williamson, E.A., Muller, E., Said, J.W., Heber, D., Possinger, K., and Koefler, H.P. *UCLA, CSMC, Los Angeles, CA USA and Charite', Humboldt University, Berlin, Germany.*

Our recent data showed that the combination of PPAR γ specific ligand, troglitazone (TGZ) and RAR specific ligand, ATRA synergistically and irreversibly inhibited the growth and induced apoptosis of MCF7 breast cancer cells, associated with a dramatic decrease of their bcl-2 protein levels (Elstner et al., PNAS, 95, 1998). Because of these encouraging results, we studied five additional breast cancer cell lines (BT474, SKBR3, T47D, MDS-MB-231, MDA-MB-436) having a variety of biological profiles. Our data showed that all of these lines expressed PPAR γ . TGZ ($\leq 10^{-5}$ M) alone slightly inhibited their clonal growth. ATRA by itself (10^{-6} M) inhibited only estrogen positive lines (BT474, T47D). Combination of both ligands additively inhibited the growth of all breast cancer lines except SKBR3. Notably, the highly tumorigenic MDA-MB-231 cells underwent massive apoptosis (46%) as measured by TUNEL analysis. Interestingly, these cells expressed the highest level of bcl-2 compared to other breast cell lines, as measured by Western blotting. After treatment with either TGZ or ATRA, no significant effect was observed on levels of bcl-2, but the combination of both ligands down-regulated this protein by 50%. Taken together, the combination of TGZ and ATRA decreased clonal growth of four of 5 breast cancer cell lines and one of them underwent apoptosis with a concomitant decrease of bcl-2 levels.

#2518 CAG repeats of the androgen receptor gene in breast cancer and its association with survival. Yu H¹, Bharaj B², Gai M³, Diamandis EP². (¹Louis Medical Center, Shreveport, LA 71130; ²Mount Sinai Hospital, Toronto, Ontario M5G 1X5; ³University of Turin, Turin, Italy).

Androgen receptor (AR) mediates the action of androgen and the AR gene contains a series of CAG trinucleotides. Studies have shown that the CAG repeats are associated with prostate cancer risk and progression. The role of the CAG repeats in breast cancer remains unknown. In this study, we measured the CAG repeats in breast cancer tissue using a PCR method. Of the 133 patients, 102 were heterozygous and 31 were homozygous. The mean CAG repeats for homozygous women was 21 and for heterozygous women the number was 20 in the short allele and 24 in the long allele. The length of CAG repeats either in one allele or in both alleles was inversely correlated with histological grade ($r = -0.23$ or -0.26 , $p < 0.05$). An association between positive lymph nodes and fewer CAG repeats in both alleles was also suggested ($p = 0.06$). Survival analysis showed that the total number of CAG repeat in both alleles was associated with patient overall survival. With every CAG repeat increase, there was a 6% reduction in the risk of death ($RR = 0.94$, $p = 0.03$). However, the association became no longer significant when clinical and pathological variables were adjusted in the analysis. This change in significance could be due to the reduction of sample size in the multivariate analysis. In summary, the results of our study suggest that longer CAG repeats may be involved in less aggressive cancer and that the CAG repeats may play a role in breast cancer progression.

#2519 Cell-transforming activity and genotoxicity of 2-methoxyestradiol in cultured Syrian hamster embryo cells. Tsutsui, T., Tamura, Y. and Barrett, J.C. *The Nippon Dental University, Tokyo 102-8159, Japan and National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.*

2-Methoxyestradiol (2-MeOE $_2$) is an endogenous metabolite of estradiol and an inhibitor of microtubule assembly. Other microtubule inhibitors, e.g., Colcemid or diethylstilbestrol have transforming and genotoxic activities in cultured mammalian cells. To assess the *in vitro* carcinogenicity and related activity of 2-MeOE $_2$, the abilities of this metabolite to induce cellular transformation and genetic effects were examined using the Syrian hamster embryo (SHE) cell model. Cell growth was reduced by treatment with 2-MeOE $_2$ at 0.1–1.0 μ g/ml in a dose-related manner. Treatment with 2-MeOE $_2$ at 0.03–0.3 μ g/ml for 48h induced a dose-dependent increase in morphological transformation of SHE cells. A statistically significant level of gene mutations at the Na⁺/K⁺ ATPase locus or the *hprt* locus was also observed in cells treated for 48h with 2-MeOE $_2$ at 0.1 or 0.3 μ g/ml, respectively. Treatment of SHE cells with 2-MeOE $_2$ at 0.1–1.0 μ g/ml for 24h elicited chromosome aberrations, the type of which was mainly breakage and pulverization. Numerical chromosomal changes were also detected both in the near diploid range and in the tetraploid and near tetraploid ranges. Our results indicate that 2-MeOE $_2$ has cell-transforming and genotoxic activities in cultured mammalian cells.

#2520 Taxol resistance and overexpression of TRAF1 and cIAP2 in ER α negative breast cancer cells with constitutive NF- κ B activity. Patel, N.M., Newton, T.R., Stauss, C.R., Rice, S., Goulet, R., Jr., Nakshatri, H. *Departments of Surgery, Medicine, Biochemistry and Molecular Biology, and Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202.*

The transcription factor NF- κ B is involved in the activation of genes important for tumor invasion, metastasis and chemotherapeutic resistance. Normally, NF- κ B is sequestered in the cytoplasm in an inactive state by inhibitory I κ B proteins. It has been shown earlier that there is constitutive NF- κ B activity in ER α negative breast cancer cells. In this study, we have investigated the role of NF- κ B in chemotherapy resistance, in particular to taxol. We generated MDA-MB-231 cells that overexpressed superrepressing I κ B α (I κ B α SR). Using flow cytometry, trypan blue exclusion, caspase 3 activation and clonogenic assays, we noted

APPENDIX 29

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Altered expression of estrogen receptor alpha variant mRNAs
between adjacent normal breast and breast tumor tissues.

Inabis 98. 5th Internet World Congress for Biomedical Sciences.
<http://www.mcmaster.ca/inabis98/index.html>. Abstract 0756, 1998.

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Altered Expression of Estrogen Receptor Alpha Variant mRNAs Between Adjacent Normal Breast And Breast Tumor Tissues.

AbstractIntroduction

Leygue, E (*Dept. Biochem. and Mol. Bio., University of Manitoba,
Canada*)

Materials & Methods

Dotzlaw, H (*Dept. Biochem. and Mol. Bio., University of Manitoba,
Canada*)

ResultsDiscussion &
Conclusion

Watson, PH (*Dept. Pathology, University of Manitoba, Canada*)
Murphy, LC (*Dept. Biochem. and Mol. Bio., University of Manitoba,
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References

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Discussion
Board

Abstract

Several variant forms of the estrogen receptor alpha (ER-alpha) mRNA have been described in human breast tissues. Among these, ER-alpha mRNA variants truncated after sequences encoding exon 2 of the wild-type ER-alpha mRNA (ERC4 mRNA), or deleted in exon 3 (ERD3 mRNA) or exon 5 (ERD5 mRNA) sequences, were previously shown to be differentially expressed between independent normal and breast tumor tissues. Using semi-quantitative reverse transcription-polymerase chain reaction assays, we have investigated the expression of these variant mRNAs relative to wild-type ER-a mRNA in 18 samples of normal breast tissues and their adjacent matched breast tumor tissues. A general trend towards a higher ERC4 mRNA and a lower ERD3 mRNA relative expression in the tumor compartment was observed. These differences reached statistical significance when considering only the ER-positive/progesterone receptor positive ($p=0.019$) and the ER-positive ($p=0.023$) subsets, as measured by the ligand binding assay, respectively. A significantly ($p=0.035$) higher ERD5 mRNA relative expression was observed in tumor components overall. These data confirm previous observations and demonstrate that changes in the expression of ER-alpha variant mRNAs relative to wild-type occur between adjacent normal and neoplastic breast tissues.

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Poster Number PAleygue0756

Keywords: estrogen receptor, breast cancer, tumor progression, variant mRNA

ABSTRACT

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APPENDIX 30

Leygue E, Hall R, Dotzlaw H, Watson PH, and Murphy LC

Conserved patterns of estrogen receptor- α variant mRNA expression in primary human breast tumors and matched lymph node metastases.

21th Annual Breast Cancer Symposium.
San Antonio, Texas, Abstract 564, 1998.

- 561 CORRELATION BETWEEN MORPHOLOGIC PARAMETERS OF VASCULARISATION, COLOR DOPPLER IMAGE FEATURES (CD) AND LYMPH-NODE METASTASIS IN BREAST CANCER. Blohmer JU, Gohlke A, Hufnagel P, Schneider U, and Lichtenegger W, University Charité, 10117 Berlin, Germany.

The goal of the current study was to determine possibly significant correlations between various morphological parameters of vascularisation and semiquantitative CD findings (pixel classes) and lymphnode metastasis (pN1).

34 patients (P) with an invasive ductal breast carcinoma (BC) (50% pT1) were investigated pre-operatively by means of CD. The BC, which were selected for a homogeneous blood flow, were assigned on the basis of the CD image to one of 4 pixel classes. An area with clear invasion was stained using CD-34 antibodies. An automated microscopic image analysis was then employed to determine various morphometric parameters of blood vessels.

For large vessels (diameter > 100 µm) there were sign. (t-test, $p < 0.001$) differences between the pixel class groups with regard to the no. of vessels, the total vessel cross-sectional area (no. of vessels x mean vessel lumen). For small vessels (< 100 µm) there were sign. ($p < 0.03$) differences between the pixel class groups with regard to the mean vessel wall thickness, the mean vessel lumen, the mean outer vessel cross-section and the total vessel cross-sectional area. For pN1 BC the mean vessel lumen and mean total vessel cross-sectional area of the large vessels, the mean vessel lumen and outer vessel cross-section of the small vessels were significantly ($p < 0.04$) larger than for pN0 BC.

CD can be used to draw conclusions about various morphological features of vascularization. It can be used to show blood supply in smaller blood vessels. The vascularization of a BC (based on CD and morphologic features) permits conclusions to be drawn as to the metastatic lymph-node status of a patient.

- 563 MODULATION OF MAMMARY TUMOR DEVELOPMENT BY A MIXTURE OF ORGANOCHLORINES FOUND IN HUMAN MILK. Desautels D¹, Leingartner K¹, Perkins G², Archer MC³, Yang J¹, Wade M¹, Yagminas A¹. ¹Environmental and Occupational Toxicology, Health Canada; ²Ottawa Univ./Civic Hospital, Ottawa, ON, Canada. ³Univ. of Toronto, ON, Canada.

Epidemiological studies remain controversial with respect to identifying a link between organochlorine (OC) exposure and breast cancer. Exposure to OCs, such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCB) during critical periods of development, is hypothesized to increase the risk of developing breast cancer. Thus, our objective was to determine if the most abundant and toxic OCs found in human milk, ingested during the neonatal period, are modulating the development of mammary tumors in the rat. A mixture composed of DDT, its major metabolite, dichlorodiphenyldichloroethene (DDE), and 19 PCB congeners, was prepared according to their median concentrations found in the milk of Canadian women. *In vitro*, our results demonstrated that the mixture, at a concentration 5000 times higher than the human milk level, stimulated the proliferation of breast cancer cells (MCF-7). *In vivo*, the mixture administered by gavage to neonatal rats from Day 1 to 21 days of age induced hepatic microsomal enzymes (ethoxy- and benzyloxy- (CYP-1A1, -2B/3A, $p < 0.05$) and pentoxy- resorufin-o-deethylase activities (CYP-2B, $p > 0.05$)). There were no indications of synergistic or additive effects between different categories of OCs on hepatic microsomal enzyme activities. Mammary tumor development was studied in rats treated with nitrosomethylurea (NMU) at 21 days of age, then exposed as above, to the mixture at doses of 10 to 1000 times the amount consumed by a human infant. The highest dose delayed ($p = 0.04$) the development of mammary tumors. In contrast, all doses of the mixture, as well as 2.5 µg/kg b.w. of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) given in a single dose at 18 days of age prior to NMU treatment at 21 days, increased the percentage of rats developing mammary tumors when they reached more than 200 days of age. Although the animal phase is still to be completed, this latter effect is statistically significant only for the TCDD treated rats. This preliminary data demonstrates dose dependent and temporal effects of OCs on mammary tumor development in the rat.

- 562 IDENTIFICATION OF TAMOXIFEN-DNA ADDUCTS IN WHITE BLOOD CELLS FROM BREAST CANCER PATIENTS. Umemoto A¹, Shibutani S², Komaki K¹, Suwa M¹, Lin C¹, Minura S¹, and Monden Y¹. ¹Second Department of Surgery, University of Tokushima, Tokushima 770-8503, Japan, ²Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, NY 11794, USA, ³R. & D. Division, Nippon Kayaku, Co., Ltd., Tokyo 115-8588, Japan.

The increased risk of endometrial cancer in breast cancer patients taking tamoxifen has been indicated in several large trials. The formation of DNA adducts by tamoxifen may be one cause. Tamoxifen-DNA adducts in white blood cells from breast cancer patients treated were analyzed by ³²P-postlabeling coupled with a novel HPLC system that gives a minimum detection limit of 3.4 adducts/10¹⁰ nucleotides.

White blood cells were collected from 30 tamoxifen-treated patients (20 mg/day, 3 months minimum) and 6 control patients. To identify tamoxifen-DNA adducts, the retention time on HPLC was compared to synthetic standards, alpha-(N²-deoxyguanosinyl)tamoxifens prepared by reacting alpha-sulfate tamoxifen with dG 3'-monophosphate. Tamoxifen-DNA adducts were detected in 11 of 30 patients by combining two types of HPLC columns: the level of adducts was 1.83 ± 1.08 adducts/10⁹ nucleotides. No tamoxifen-derived adducts were observed in control patients. This tamoxifen-DNA adduct detected in patients is detectable as a second major adduct in the liver of rats given tamoxifen.

If the tamoxifen-DNA adduct found in white blood cells is also formed in uterine tissues, this may cause critical mutations leading to endometrial cancer.

- 564 CONSERVED PATTERNS OF ESTROGEN RECEPTOR- α (ER- α) VARIANT mRNA EXPRESSION IN PRIMARY HUMAN BREAST TUMORS AND MATCHED LYMPH NODE METASTASES. Leygue E, Hall RE*, Dotzlaw H, Watson PH and Murphy LC. University of Manitoba, Winnipeg, Manitoba, Canada, R3E 0W3, and Flinders University of South Australia, Bedford Park, S.A., Australia*.

Previously we showed that the relative expression of clone 4 ER- α variant mRNA (C4ER- α) was increased in axillary node positive primary breast tumors compared to node negative tumors. We have now examined the relative expression of C4ER- α , exon 5 deleted and exon 7 deleted ER- α variant mRNAs in 15 primary breast tumors and their matched synchronous axillary lymph node metastases.

The pattern of all exon deleted ER- α variant mRNAs was determined using a long range RT-PCR approach. The pattern of deleted ER- α mRNAs expression between any one primary tumour and its matched lymph node metastasis was conserved. The measurement of the relative expression of specific exon deleted ER- α variant mRNAs (exon 5 deleted and exon 7 deleted ER- α) was done using a previously validated semi-quantitative PCR approach. C4ER- α mRNA was measured using a modified version of a previously described TriplePrimer-PCR assay. There were no statistically significant differences in the relative expression of the exon deleted ER- α or C4ER- α mRNAs between primary and concurrent metastatic tumors. This extends our previous data on ER- α variant expression and progression in breast cancer. We previously showed that ER- α variant expression is altered during breast tumorigenesis and is correlated with some prognostic markers in primary breast tumors. The current data show that both the pattern and level of expression of ER- α variants are conserved between matched primary breast tumors and their concurrent lymph node metastases. Thus altered ER- α variant expression, a possible marker of altered ER- α signalling and breast cancer progression, likely occurs before breast cancer cells acquired the ability to metastasize.

APPENDIX 31

Dotzlaw H, **Leygue E**, Watson PH, and Murphy LC

Expression of estrogen receptor- α (ER- α) and estrogen receptor-beta (ER- β) mRNA in primary human breast tumors and matched

normal human breast tissue samples.

10th International Congress of Hormonal Steroids.
Quebec, Quebec, Abstract 63, 1998.

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EXPRESSION OF ESTROGEN RECEPTOR- α (ER- α) AND ESTROGEN RECEPTOR- β (ER- β) mRNA IN PRIMARY HUMAN BREAST TUMORS AND MATCHED NORMAL HUMAN BREAST TISSUE SAMPLES. H Dotzlaw, E Leygue, PH Watson, LC Murphy*. Department of Biochemistry & Molecular Biology and Dept of Pathology, University of Manitoba, Winnipeg, MB, Canada. X

Using an RT-PCR approach we have recently detected ER- β mRNA in some human breast tumor samples. We have now confirmed this using RNase Protection Assays. The ER- β mRNA levels varied amongst tumors and were not correlated with ER- α mRNA levels, but several tumors co-expressed ER- α and ER- β mRNA. We have also identified ER- β mRNA in normal breast tissue samples. The data suggest that ER- β has a role in human breast tissues. To gain further insight into the possible role of each ER in human breast tissues we compared the expression of ER- α and ER- β mRNA in 18 breast tumors and their matched adjacent normal breast tissues. Eleven samples were from women whose breast tumor was ER+ by ligand binding assay (> 3 fmol/mg protein), and 7 samples were from women whose breast tumor was ER- by ligand binding assay. A multiplex RT-PCR assay was developed to measure relative levels of ER- α and ER- β mRNA simultaneously in the same assay. The PCR products were labelled with P³²-dCTP and the two products representing ER- α (148 bp) and ER- β (241 bp) cDNA were separated on 6% PAGE/7M urea. The gels were dried, exposed to XAR Film and the bands corresponding to ER- α and ER- β cDNA excised and counted. The ratio for ER- α /ER- β was determined in each sample and the mean of three separate PCR reactions was obtained. The ratio of ER- α /ER- β varied widely amongst both tumors and normal tissue samples. However, in tissue from women with ER+ tumors the ER- α /ER- β ratio was significantly greater in tumors versus their matched normal tissues ($P < 0.02$, Wilcoxon). This effect was due mainly to an increased expression of ER- α mRNA in tumors. Interestingly, ER- β mRNA levels in tumor but not normal tissues were positively correlated with the degree of inflammation suggesting that infiltrating lymphocytes may contribute to the overall ER- β signal in tumor samples. These data suggest that the relative roles of ER- α and ER- β in human breast tissues change during breast tumorigenesis.

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ISOLATION OF GENES REGULATED BY ANDROGENS IN BREAST CANCER CELLS. J. Lapointe* and C. Labrie. Laboratory of Molecular Endocrinology, CHUL Research Center and Laval University, Quebec, Canada. X

The androgen receptor is expressed in 50 to 90% of breast tumors and androgens or androgenic compounds have been shown to inhibit breast cancer growth in women. A direct growth-inhibitory effect of androgens has been demonstrated in estrogen-responsive ZR-75-1 human breast cancer cells but little is known regarding the mechanism of action of androgens on breast cancer cell growth. In this study we have used differential display PCR to identify androgen-regulated genes in breast cancer cells. ZR-75-1 cells were grown for 48 hours in the presence or absence of 1 nM dihydrotestosterone (DHT). RNA was then extracted and used for differential display. We have identified a DHT-induced ≈ 0.6 kb fragment by differential display PCR. This radiolabeled DNA fragment recognized a mRNA of approximately 3 kb that is induced strongly in DHT-treated ZR-75-1 cells. Further characterization of this androgen-regulated gene should provide new insights into the mechanisms of androgen action in breast cancer.

APPENDIX 32

Lu B, **Leygue E**, Dotzlaw H, Watson PH, Murphy LJ, and Murphy LC

Estrogen receptor alpha and beta (ER- α and ER- β) mRNA variants in human and murine tissues: species specific differences.

80th Annual Meeting of the Endocrine Society.
New Orleans, Louisiana, Abstract P1-574, 1998.

the rat ER β shares homology with ER α in the AF2 region, we also hypothesized that TERP-1 would have similar effects on ER β . In the same transfection system, TERP-1 enhanced ER β activation of a reporter gene up to 5-fold in the presence of 10nM estradiol. Furthermore, TERP-1 enhanced FSK and PMA induced transcription from ER β an additional 50% over stimulated levels. These data further support a role for TERP-1 in enhancing estrogen receptor-induced transactivation by activated estrogen receptors.

P1-572

THE TRUNCATED ESTROGEN RECEPTOR α (ER α) VARIANT, TERP-1, SUPPRESSES ER α TRANSCRIPTIONAL ACTIVATION AND BINDING TO DNA.

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The amino-terminally truncated, rat pituitary ER α variant, TERP-1, contains a unique 5' end fused to exons 5 through 8 of ER α . TERP-1 is regulated throughout the estrous cycle. RNase protection assays demonstrated that TERP-1 mRNA is undetectable at late estrus, but becomes the primary ER α isoform at proestrus, when the ratio of TERP-1 to ER α was at least 2.5 to 1. In transfected cells, TERP-1 influences transcription of estrogen responsive genes only with concomitant ER α expression. At high cellular TERP-1 to ER α ratios, TERP-1 significantly suppresses transactivation by ER α below basal levels. We investigated possible mechanisms for the suppressive effects of TERP-1 on ER α activity by determining TERP-1's cellular location by immunofluorescence in transfected ER negative Cos cells, and examining TERP-1's effects on the binding of ER α to the vitellogenin estrogen response element (ERE) *in vitro*. For immunofluorescence studies, we epitope-tagged TERP-1 with the FLAG sequence to differentially recognize TERP-1 and used ER α antibodies which recognized both receptor forms or only ER α . In transfected Cos cells, ER α remains primarily in the nucleus in the absence of estrogen. In contrast, TERP-1 is predominantly cytoplasmic without estrogen and ER α expression, with minimal TERP-1 staining in the nucleus. In the presence of ER α and estrogen, TERP-1 is equally distributed in the cytoplasm and nucleus. The presence of TERP-1 in the nucleus suggests the protein may have a direct effect on ER α transactivation. Gel shift analyses corroborate the transcription and immunofluorescence data. Increasing amounts of *in vitro* translated TERP-1 protein were incubated with a constant amount of ER α protein and ³²P-labeled oligonucleotides containing one copy of the ERE. The ratio of TERP-1 to ER α protein in the incubations increased from 1:10 to 3:1. Increasing amounts of TERP-1 protein inhibited ER α binding to the ERE and could eliminate ER α binding at highest TERP-1 concentrations. This effect was specific, as the addition of other nuclear proteins, without TERP-1, did not inhibit ER α binding to the ERE. TERP-1's inhibitory actions, which include its ability to suppress both ER α binding to the ERE and ER α transactivation, are like those of the structurally similar, inhibitory nuclear hormone receptor, SHP. These data suggest that the high concentrations of TERP-1 which are found to inhibit the actions of ER α in the pituitary, may have physiological relevance to the rat reproductive cycle.

P1-573

FUNCTIONAL ANALYSIS OF AN ISOFORM OF ESTROGEN RECEPTOR β (ER β). W D Pennie,* T C Aldridge, N Macdonald, S Stone, A N Brooks. Molecular Endocrinology, Zeneca CTL, Alderley Park, Macclesfield, Cheshire, UK

We have obtained the complete cDNA sequence of an isoform of rat ER β which has an 15 amino-acid insert in the ligand binding domain (ER β 2; ²²SSDPHWHVAQMKSAAPR³³⁸). The ability of ER β 2 to transcriptionally activate a reporter plasmid (consisting of a consensus vitellogenin ERE cloned upstream of a minimal SV40 promoter driving luciferase) when transiently transfected into Cos-1 cells was examined. The reporter in cells transfected with ER β alone was activated in a dose-dependent manner by stimulation with estradiol-17 β (10⁻¹² - 10⁻⁶ M). In contrast, estradiol failed to activate the reporter in cells transfected with ER β 2 alone. When cells were co-transfected with a constant amount of ER β and increasing amounts of ER β 2 there was a significant decline in reporter activation by estradiol. Using *in situ* hybridization analysis, we have shown high expression of ER β 2 and ER β in the prostate, ovary and pituitary gland. Semi-quantitative RT-PCR revealed a 10 fold excess of ER β over ER β 2 mRNA in the prostate, whereas in the pituitary gland the mRNAs for the two receptors were found in equal amounts. Finally, *in vitro* translated ER β and ER β 2 were both found to be capable of binding to the vitellogenin estrogen response element in gel shift assays. These data provide evidence for an isoform of ER β which may act as a negative regulator of ER β function. The differences in relative abundance of these two receptors in different tissues may play a role in determining tissue-specific responses to estrogenic hormones.

P1-574

ESTROGEN RECEPTOR ALPHA AND BETA (ER- α and ER- β) mRNA VARIANTS IN HUMAN AND MURINE TISSUES: SPECIES SPECIFIC DIFFERENCES. B Lu,* E Leygue,¹ H Dotzlaw,¹ P H Watson,² L J Murphy,² L C Murphy.¹ ¹Depts. of Biochemistry and Molecular Biology, ²Physiology, ³Pathology, University of Manitoba, Winnipeg, Manitoba, Canada

Estrogens are major regulators of growth and differentiation in a range of tissues, particularly in mammary gland development, as well as the tumorigenesis and progression of mammary tumors. With the recent identification of ER- β in both human and murine mammary tissue, estrogen action in mammary tissue likely involves both ER- α and ER- β . Multi-faceted nature of estrogen action is further implied by the previous identification of numerous ER- α splice variants, at least, in human tissue. It was of interest to determine if similar splice variants existed for ER- β , and if so, compare the expression of ER- α and ER- β splice variants in human and murine tissues.

ER- β mRNA splice variants were identified in human breast tumors as well as both human and murine ovarian, uterine and mammary tissues. In both species mRNAs deleted in exons 5 or 6 or exons 5+6 were characterized by RT-PCR followed by cloning and sequencing. In mouse tissues, an ER- β transcript containing 54 nucleotides inserted inframe between exons 5 and 6 was identified. No equivalent of the mouse inserted transcript was detected in any of the human tissues analyzed. In human tissues, ER- α RNAs corresponding to deletions in exon 3, exon 4, exon 5, exon 7 or exon 3 + 4 were identified. In contrast to what was observed in human tissues, only an exon 4 deleted ER- α mRNA variant was detected at apparently low levels in all murine tissues analyzed. In summary, both ER- α and ER- β splice variants are expressed in both human and murine tissues. Species specific differences were apparent in either the type and/or the level of expression of ER- α and ER- β RNA splice variants.

P1-575

DETERMINATION OF ER α / β HETERODIMER ACTIVITY *IN VIVO*. Gilles B Tremblay,*¹ Andre Tremblay,¹ Fernand Labrie,² Vincent Giguere.¹ ¹Molecular Oncology Group, Royal Victoria Hospital, Montreal, QC, Canada, ²Laboratory of Molecular Endocrinology, CHUL Research Center, Quebec, QC, Canada

The identification of a second estrogen receptor, termed ER β , has caused a dramatic change in the way we view estrogen physiology. The contribution of each receptor in the regulation of estrogenic responses is only beginning to be explored and it is unclear whether ER α or β , if either, will predominate in a given system. While these receptors can function as homodimers, it has recently been reported that they can form heterodimers *in vitro*. However, the relative contribution of each partner to the activity of the heterodimer after estrogen or growth factor induction is unknown. We have addressed this question by generating a system to specifically measure ER α / β heterodimers in transfected cells. Mutations were created in the DNA-binding domain of ER β that change its DNA-binding activity from ERE (AGGTCA) to glucocorticoid response element (GRE) (AGAACA) specific. This mutant, termed ER β _{GR}, when co-transfected with the wild type ER α , forces the activity of ER α / β heterodimers in the presence of a hybrid element containing an ERE and a GRE half-site spaced by three base-pairs. The heterodimer causes the expression of a luciferase reporter gene preceded by this hybrid element in the presence of estradiol and is inhibited by antiestrogens. The system described above allows the measurement of the activity of ER α / β heterodimers *in vivo* exclusively without the interference of ER α and ER β homodimers and permits us to quantitate the relative contribution of both AF-1 and AF-2 functions within the context of the ER α / β heterodimer.

P1-576

THE AFI DOMAIN OF ER β BUT NOT ER α CONTRIBUTES TO LIGAND INDEPENDENT TRANSACTIVATION BY SRC-1. Andre Tremblay,*¹ Gilles B Tremblay,¹ Fernand Labrie,² Vincent Giguere.¹ ¹Molecular Oncology Group, Royal Victoria Hospital/McGill University, Montreal, QC, Canada, ²Laboratory of Molecular Endocrinology, CHUL Research Center, Quebec City, QC, Canada

We previously demonstrated that the steroid receptor coactivator-1 (SRC-1) up-regulates mouse ER α and ER β activity in the presence of estradiol whereas ER β is induced in the absence of ligand (Mol.Endo.11:353-365,1997). Here, we show that the ligand independent effect of SRC-1 on ER β transactivation occurs in a dose-dependent manner using an ERETKLuc or a PS2Luc reporter in transfected COS-1 and HeLa cells. The activation function 2 (AF2) domain of ER β is not involved in this activation since the pure antiestrogens ICI182,780 and EM-652, but not OHT, which is AF2 specific, abolished the effect. This observation is supported by the use of an AF2 ER β mutant which can still be induced by SRC-1 in the absence of ligand. By using truncated forms of ER β in transient transfections, only the construct ABC but not CDEF was activated by SRC-1 in the absence of ligand, suggesting that the AB region of ER β is involved in such activation. In addition, we found that the SRC-1 activation through AB β could be transferred to ER α . A chimera consisting of AB β fused to a CDEF α fragment was responsive to SRC-1 in absence of ligand in transfected COS-1 cells with ERETKLuc reporter, while ER α was not. A similar result was also obtained with an AB β -gal4DBD construct used in transfected COS-1 cells with UASTKLuc reporter. Generation of both N- and C-terminal deletions within AB β has delineated the ligand independent effect of SRC-1 to region between amino acids 38 to 64 in ER β . SRC-1 was also able to activate GR in the absence of ligand. Interestingly, only GR showed homology with ER β in that region as opposed to the other nuclear receptors tested. These data suggest that SRC-1 could contribute to the basal activity of a number of steroid receptors in absence of ligand and that effect is mediated through a conserved motif of the AFI domain.

P1-577

CROSS INHIBITION OF BOTH ESTROGEN RECEPTOR α AND β PATHWAYS BY EACH DOMINANT NEGATIVE MUTANT. Sumito Ogawa,*¹ Satoshi Inoue,^{1,2} Akira Orimo,¹ Takayuki Hosoi,² Yasuyo Ouchi,² Masami Muramatsu.¹ ¹Dept of Biochemistry, Saitama Medical School, Saitama, Japan, ²Dept of Geriatrics, The University of Tokyo, Tokyo, Japan

Both estrogen receptor α (ER α) and recently identified ER β are nuclear receptors that are activated by estrogen. It was reported that ER α and ER β form heterodimers. Here, we show that they activate transcription rather independently than synergistically via estrogen response element (ERE). To show the cross-talk between ER α and ER β , we utilized dominant negative mutants of ERs constructed by C-terminal truncation. Interestingly, ER α -530 inhibited transactivation not only by ER α but also by ER β , whereas ER β -481 inhibited transactivation by ER α as well as by ER β . GST pull-down assay also demonstrated the cross-interaction of these mutants with wild-type ER α and ER β . Thus, we found dominant negative mutants that block both ER α and ER β signaling pathways.

APPENDIX 33

Murphy LC, **Leygue E**, Dotzlaw H, Lu B, Coutts, A.,
Huang A, and Watson PH

Multiple facets of the estrogen receptor (ER) in breast cancer.
Breast and prostate cancer, Keystone Symposium.
Copper Mountain, Colorado, 1998.

Multiple Facets of the Estrogen Receptor (ER) in Breast Cancer.

Leigh Murphy, Etienne Leygue, Helmut Dotzlaw, Biao Lu, Amanda Coutts, Aihua Huang, Peter Watson*. Departments of Biochemistry & Molecular Biology, and Pathology*, University of Manitoba, Winnipeg, MB, R3E0W3, Canada.*

Estrogens are major regulators of normal mammary gland development, as well as the growth and progression of mammary cancers. However, during breast tumorigenesis and progression, a series of poorly understood alterations in estrogen signal transduction occurs, which underlies the progression of human breast cancer from hormone dependence to independence with the accompanying development of resistance to endocrine therapies. The principal mechanism by which the effects of estrogen are mediated in either normal or neoplastic target cells is via an initial interaction with ER. Until recently it was thought that only one form of ER was involved in regulating the ER signal transduction pathway. This is no longer true. Not only have several alternatively spliced variants of the classical ER- α been described but a second ER gene (ER- β) has also been identified. ER- α , several ER- α variants as well as ER- β and ER- β variants have been detected, at least, at the mRNA level, in both normal and neoplastic human breast tissues. Alterations in the relative expression of several of these ER-like molecules occur during human breast tumorigenesis and breast cancer progression, suggesting a possible role(s) in altered ER signal transduction during breast tumorigenesis and progression. Supported by grants from NCIC, CBCRI, MRC and USAMRMC.

APPENDIX 34

Dotzlaw H, **Leygue E**, Watson PH, and Murphy LC

Expression of the estrogen receptor-beta (ER- β)
in some human breast cancer biopsy samples.

79th Annual Meeting of the Endocrine Society.
Minneapolis, Minnesota, Abstract P1-520, 1997.

P1-517

IRS-1 SIGNALING AND INSULIN-INDUCED MODULATION OF ESTROGEN RECEPTORS IN BREAST CANCER. S.Andò¹, M.Salerno¹, D.Sisci², L.Mauro¹, M.K.Nolan, E.Surmaz.
¹Department of Cellular Biology, Faculty of Pharmacy and ²Health Center, University of Calabria, CS, Italy; Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107.

The coordinate action of steroid hormones and polypeptide growth factors regulates the growth of breast cancer cells *in vitro*. While the mechanism of this synergy is not fully understood, a critical role for a cross-talk between steroid and growth factor system has been suggested; i.e. steroids modulate expression of growth factors and/or growth factor receptors, and reciprocally, growth factors affect the synthesis and the phosphorylation status of steroid hormone nuclear receptors.

We have previously shown that in MCF-7 cells, insulin up-regulates estrogen receptor (ER) content and ER binding capacity, possibly through the stimulation of tyrosine phosphorylation status of ER. The insulin receptor (IR) signaling involves several cellular substrates. One of the major substrate, a docking protein IRS-1, is indispensable for mitogenic activity of the IR. In this study we investigated whether the IRS-1 signaling pathway is also required for the insulin-induced function of ER. We developed two MCF-7-derived cell lines in which the levels of IRS-1 were down-regulated by 80% by the expression of an IRS-1 antisense RNA (measured by Western blotting). The MCF-7/anti-IRS-1 cells displayed pronounced growth inhibition in monolayer and soft agar cultures and increased rate of apoptosis in phenol-red free serum-free medium. The ER protein content was similar in MCF-7 cells and in MCF-7/anti-IRS-1 clones. However, ER binding capacity was markedly (by at least 2.7-fold) elevated in MCF-7/anti-IRS-1 cells compared with that in MCF-7. Insulin up-regulated ER levels and ER binding capacities in all tested cell lines, with more pronounced effects in MCF-7/anti-IRS-1 clones. Interestingly, in MCF-7/anti-IRS-1 clones, the basal level of tyrosine phosphorylation was higher (2-fold) than that in MCF-7 cells. Insulin treatment enhanced tyrosine phosphorylation of cellular proteins in all examined cells, however, the extent of stimulation was greater in MCF-7/anti-IRS-1 cells.

These data suggest that in MCF-7 breast cancer cells, IRS-1-dependent pathway plays a positive role for cell proliferation and survival but is not required for insulin-dependent up-regulation of ER. In fact, since in MCF-7/anti-IRS-1 cells, basal ER levels and IR-stimulated ER binding were elevated compared with that of MCF-7 cells, IRS-1 may be involved in negative regulation of ER in breast cancer cells.

P1-518

AROMATASE EXPRESSION IN CELLS ISOLATED FROM THE NORMAL BREAST. B.J.Long*, Q. Lu, C. Rodriguez-Valenzuela, and A.M. Brodie. Dept. of Pharmacology, School of Medicine, University of Maryland, Baltimore, MD 21201.

Estrogens have important roles stimulating the progression and growth of human breast cancers. We have recently reported aromatase expression in the tumor epithelial cells of breast cancers by immunocytochemistry, and in the normal breast we observed aromatase expression in the epithelial cells of the terminal ductal lobular units (TDLU), the putative cells of origin of breast cancers, as well as the surrounding stromal cells. In the present study we have isolated normal luminal epithelial cells (LEC), myoepithelial cells (MEC), and fibroblasts (FIB) from the TDLUs of three women (14, 17 and 22 years old), who had undergone mammary reduction surgery. Cells were isolated using selective media and, depending on cell type, could be passaged between 5 and 20 times before senescence. We have measured aromatase activity levels and expression of aromatase mRNA by RT-PCR. Aromatase activity and mRNA were detected in all the cells from the 14 and 22 year old patients, and with the 14 year old patient, activity was highest in the LEC. In the LEC and MEC from the 22 year old patient, aromatase activity could be inhibited by the aromatase inhibitor, 4-hydroxyandrostenedione. Each cell type from the 17 year old patient expressed aromatase activity and mRNA, and we examined aromatase activity levels following treatment with dexamethasone (Dex), cAMP, and phorbol ester (PDA). Activity, which was highest in the FIB was modestly stimulated by cAMP alone and significantly stimulated by a combination of cAMP plus PDA. These effects were observed in the presence and absence of serum. MEC, which were selected in serum-free medium (SFM), had reduced activity in the presence of serum, but did not respond to any treatments regardless of serum status. LEC, which were selected in medium containing 10% serum, had reduced activity in SFM, and in this media aromatase activity was stimulated by Dex, cAMP and a combination of cAMP and PDA. In the LEC isolated from the 14 year old patient, activity was stimulated only by cAMP. These results show that normal breast epithelial cells express aromatase, suggesting that this local estrogen production may have an impact on the growth of mammary cells, particularly as they progress to a malignant state. (NIH-CA-62484).

P1-519

DIETARY FATS STIMULATE PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-MEDIATED TRANSCRIPTION IN MCF7 HUMAN BREAST CANCER CELLS. S.Rai^{1,2}, PL Tate¹ and MW Kilgore^{1,2}. ¹Greenville Hospital/Clemson University Biomedical Cooperative and ²Department of Biological Sciences, Clemson, SC, 29634.

Peroxisome proliferators are a group of structurally diverse compounds that were characterized based on their ability to cause an increase in both the size and number of hepatic peroxisomes when administered to rodents. They are non-genotoxic carcinogens which exert their effects on cells by activating peroxisome proliferator activate receptor (PPAR), itself a members of the nuclear receptor family. Combining the facts that 1) some epidemiological data suggests high-fat diets are associated with an increase in breast cancer rates, 2) peroxisomal proliferators are known carcinogens acting through a nuclear transcription factor and 3) this receptor can be activated by dietary fatty acids, we set about to determine whether peroxisomal proliferators might play a role in breast disease. Previously we have demonstrated that MCF7 cells are functionally responsive to the peroxisome proliferators WY 14,643 and LY 171,883. In this report we have used a quantitative transcriptional assay to assess the effects of linoleic acid on the transcriptional activation of PPAR in MCF7 cells. As 15-deoxy- $\Delta^{12,14}$ -PGJ₂ has been described as an endogenous ligand for PPAR, indomethacin has been included and demonstrates the specificity of this response. Additionally, while ω -3 fatty acids have shown no effects ω -6 fatty acids appear to be activators of PPAR in MCF7 cells. Furthermore, Western blot analysis using two separate antibodies clearly indicates the presence of PPAR protein in these cells. Although the physiological significance of a functional PPAR in human breast cancer cells remains to be determined, these data demonstrate that MCF-7 cells are a useful model to study the effects of peroxisome proliferators such as fatty acids in the human breast.

P1-520

EXPRESSION OF THE ESTROGEN RECEPTOR-BETA (ER- β) IN SOME HUMAN BREAST CANCER BIOPSY SAMPLES. H Dotzlaw*, E Leygue, PH Watson and LC Murphy. Depts of Biochemistry & Molecular Biology and Pathology, University of Manitoba, Winnipeg, MB, R3E 0W3, Canada.

Recently cDNA for the human ER- β was cloned and characterized from testes¹. ER- β protein has similarities to the classical ER referred to as ER- α , in terms of structure and function, e.g. ER- β binds estradiol and can activate an ERE-CAT reporter gene¹ however ER- β is encoded by a gene distinct from ER- α and appears to have a different but overlapping pattern of tissue specific expression to that of ER- α . The similarities yet distinct nature of ER- β and ER- α suggest that ER- β may effect estrogen signal transduction in target cells. We have therefore investigated the expression of ER- β in human breast cancer biopsy samples. RNA was isolated from several breast cancer biopsy samples and a sample of non-malignant human testis. One ug of total RNA was reverse transcribed and an aliquot of the resulting cDNA subjected to polymerase chain reaction (PCR) using primers specific for ER- β , ER- α and glyceraldehyde-3-phosphate dehydrogenase. Aliquots of the resulting PCR products were analyzed by 1.8 % agarose gel electrophoresis and visualized by ethidium bromide staining. Using specific primers for ER- β the expected 259 bp DNA band was detected in the human testis sample as well as very faintly detected in some breast cancer biopsy samples. Increased sensitivity of detection was achieved by incorporation of P³²-dCTP into the PCR reaction, followed by 7M urea/PAGE (6 %) and autoradiography. A strong 259 bp band was detected in human testis while a similar band of reduced intensity was present in some breast cancer biopsy samples. Using primers specific for ER- α an expected 483 bp fragment was detected in several breast cancer biopsy samples by ethidium bromide staining. In conclusion ER- β expression is detectable in some human breast tumors using highly sensitive techniques. The pattern of expression appears unrelated to ER- α expression. 1. Mosselman S, Polman J, Dijkema R ER- β : identification and characterization of a novel human estrogen receptor. *FEBS Letters* 392: 49-53, 1996.

APPENDIX 35

Leygue E, Dotzlaw H, Watson PH, and Murphy LC

Expression of exon 6-deleted progesterone receptor variant mRNA
in normal human breast tissue and breast tumors.

79th Annual Meeting of the Endocrine Society. Minneapolis, Minnesota,
Abstract P1-521, 1997.

P1-521

EXPRESSION OF EXON 6 DELETED PROGESTERONE RECEPTOR VARIANT mRNA IN NORMAL HUMAN BREAST TISSUE AND BREAST TUMOURS.

E. L. Yague*¹, H. Dotzlaw¹, P. Watson², L.C. Murphy¹. ¹ Dept of Biochemistry and Molecular Biology, ² Dept of Pathology, University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, R3E0W3, Canada.

The progesterone receptor (PR) is an important prognostic marker in breast cancer as well as a marker of responsiveness to endocrine therapies. The presence of several exon-deleted PR variant mRNAs in both normal and neoplastic breast samples has recently been reported. Amongst them, a variant mRNA deleted in exon 6 (D6-PR mRNA), that if translated, would encode a truncated PR-like protein missing the hormone binding domain and one of the transactivating domains of the wild-type PR protein. In order to determine whether changes in D6-PR variant expression could occur during tumorigenesis, its expression was investigated by reverse transcription and PCR in ten normal reduction mammoplasties samples, nine breast tumors with high PR levels (>100 fmol/mg protein) and eight breast tumors with low PR levels (<15 fmol/mg protein), as determined by ligand binding assay. The relative expression of D6-PR to wild-type PR mRNA was lower ($P < 0.01$) in normal than in tumor breast samples. Moreover, a trend to lower ($P < 0.1$) relative D6-PR expression was observed in high PR tumors, compared to low PR tumors. These data suggest that increased expression of D6-PR occurs during tumor progression.

P1-522

FUNCTIONAL CHARACTERIZATION OF NOVEL INSERTED ESTROGEN RECEPTORS (ER). D. Douglas*, and L.C. Murphy. Dept of Biochemistry & Molecular Biology, University of Manitoba, Winnipeg, MB, R3E 0W3, Canada.

Previously we identified ER-like mRNAs containing inserted sequences in some human breast tumors. Cloning these ER-like mRNAs showed: 1) mRNAs containing duplication of exon 6 ER sequences. The predicted 51 kDa protein is identical to wild type ER protein upto amino acid (a.a) residue 457 followed by 5 novel a.a. and truncated in the mid-E domain; 2) a mRNA containing duplication of exons 3+4. The predicted 82 kDa protein is identical to wild type ER except the a.a encoded by exons 3+4 are duplicated; 3) a mRNA containing 69 novel nucleotides inserted between exon 5 and 6. These sequences are inframe and encode 23 novel a.a between residues 412 and 413 of wild type ER. The predicted protein is 69 kDa. To assess potential function of these putative ER-like proteins, appropriate expression vectors where 1) transcribed and translated *in vitro*, and their ability to bind ³H-estradiol determined, 2) transiently co-transfected with ERE-tk-CAT into COS-1 cells \pm estradiol (10 nM) to measure transactivation potential, and 3) transiently co-transfected with the CMV-(ERE)₂-CAT into COS-1 cells to assess DNA binding potential in whole cells. Exon 6 duplicated ER did not bind estradiol nor have transactivation activity. The whole cell promoter interference assay suggested that it could bind to an ERE. The exon 3+4 duplicated ER had reduced estradiol binding and reduced ligand activated transcriptional activity. The whole cell promoter interference assay suggested that it could bind to an ERE. The 23 a.a. inserted ER had reduced estradiol binding activity and little if any transcriptional activity. The whole cell promoter interference assay suggested that it could bind to an ERE. Expression of these novel ER-like proteins in ER positive cells may modify wild type ER signal transduction. Their expression may contribute to the progression of some breast cancers from hormone dependence to hormone independence and the development of endocrine resistance.

P1-523

CO-EXPRESSION OF VARIANT PROGESTERONE AND ESTROGEN RECEPTOR TRANSCRIPTS IN HUMAN BREAST CANCER. R.L. Balleine*, S. Hunt, C. Yeates and C.L. Clarke. Westmead Institute of Cancer Research, University of Sydney, Westmead Hospital, Westmead, NSW 2145, Australia.

There is emerging evidence that some nuclear hormone receptors exist as a number of isoforms which may differ in their functional capacity and may interact to mediate hormone responses. This may be relevant to the phenomenon of receptor positive though hormone resistant breast cancer. The aim of this study was to compare the expression of wild-type and truncated ER and PR transcripts in a series of breast cancers in order to determine the extent of variant transcript expression in tumors known to contain different levels of the hormone dependent marker pS2. PR transcripts were measured by RT-PCR in 35 PR positive tumors and ER transcripts in 24 of these cases. The majority of tumors contained alternatively spliced PR transcripts although their abundance was extremely low and only detectable on heavily overexposed Southern blots. PR transcripts lacking exons 4 and 6 were the most abundant species noted and were present in 89% and 91% of tumors respectively. Deletions of exons 2 and exon 3 were detected in 80% and 91% of tumors respectively, and multiple exon deletions, notably deletions of exons 2+3 and 5+6 were detected in over 90% of tumors. Evidence for cryptic splicing was indicated by the detection of PR transcripts lacking half of exon 4 in 97% of tumors. Exon deleted ER transcripts were markedly more abundant than truncated PR transcripts. All of the tumors contained multiple exon deleted transcripts. Specific deletions of exons 2, 4, 5 and 7 were present in the majority of tumors. The exon 7 deleted variant was the most abundant species, expressed at levels ranging from 31 - 83% of wild type ER. There was no relationship between the expression of PR and ER truncated transcripts and expression of pS2. This study has shown that multiple alternatively spliced PR and ER transcripts are commonly co-expressed in breast cancer, although the level of expression of the PR variants is very low. The expression of truncated PR and ER transcripts was not associated with expression of pS2 and the implications of these variants for hormone responsiveness in breast cancer remains to be determined.

P1-524

POLYMORPHIC CAG REPEATS IN THE ANDROGEN RECEPTOR GENE IN FEMALE BREAST CANCER. Y. Elhajj¹, L. Pinsky^{1,2,3,4,5}, M. Tinfir^{1,5}. ¹Lady Davis Institute for Medical Research, Sir M.B. Davis-Jewish General Hospital; Depts. of ²Biology, ³Human Genetics, ⁴Pediatrics and ⁵Medicine, McGill University, Montreal, Quebec, CANADA.

The human androgen receptor (hAR) is an androgen modulated, DNA-binding, transcriptional-regulatory protein that is a prototypic member of the nuclear receptor superfamily and plays a role in the regulation of breast development. Epidemiological evidence suggests that women with higher lifetime "exposures" to estrogen have a higher risk of breast cancer. Estrogen promotes and maintains breast development, and androgen is antiestrogenic. The antiestrogenicity of androgen has been documented repeatedly in certain human breast cancer lines, and in advanced breast cancer, the combination of antiestrogen plus androgen therapy is superior to that of antiestrogen alone.

The transcriptional domain of the hAR is encoded by exon 1 which contains a polymorphic CAG repeat that modulates transactivation. It has been shown that longer repeats are associated with decreased transactivation, which may affect the functional estrogen:androgen balance by generating a functional hyperestrogenicity. For this reason we have chosen to assess the size of the polymorphic CAG repeat in the AR gene in breast cancer tissue. 25 fresh frozen female breast cancer samples (50 alleles) were obtained from the Manitoba Breast Tumour Bank. Genomic DNA was isolated using TRI-Reagent and amplification of the hAR was performed using nested PCR. The PCR products were separated and sized by silver staining on a 10% polyacrylamide minigel. hAR fragments of known CAG tract size (20, 22, 24, 26, 28, 32) were used as controls.

The distribution of CAG repeats in the normal population ranges from 11-33 repeats. Only 11% have tracts larger than 25 repeats and only 1% have tracts greater than 30 repeats. Our analysis showed 35% of the alleles from the breast cancer tumour samples have 26 or more repeats and 8% have more than 30 repeats. This data suggests a significant shift in the distribution of CAG repeats in the hAR gene of breast cancer tissue. The transcriptional properties of an expressed androgen receptor corresponding to a shifted CAG repeat size could lead to relative hyperestrogenicity. We are extending these studies with Class A samples which include breast cancer tissue and matching normal breast tissue. This would address the possible somatic etiology of such a shift.

APPENDIX 36

Huang A, **Leygue E**, Snell L, Murphy LC, and Watson PH

Expression of estrogen receptor variant mRNAs and determination of estrogen receptor status in human breast cancer.

88th Annual Meeting, American Association for Cancer research (AACR).
San Diego, California, Abstract 1972, 1997.

#1970 Estradiol regulation of proliferation of T47D breast cancer cells: Alterations in p53 tumor suppressor protein and mRNA levels. Dinda, S., Khattree, N., Hurd, C., Jhanwar, S.C., and Moudgil, V.K. *Oakland University, Rochester, MI 48309, Memorial Sloan Kettering Cancer Center, New York, NY 10021*

We have monitored the expression of p53 mRNA and protein levels in T47D cells treated with physiological levels of E_2 and estrogen receptor (ER) antagonists. T47D cells proliferate maximally in medium supplemented with 5–10% fetal calf serum. Cells grown for 5–20 days in the medium containing charcoal-stripped serum (i.e. depleted of endogenous steroids) exhibit a lowered rate of proliferation (>80%). Supplementation with 1–10nM E_2 increased the rate of cell proliferation. In cells cultured in steroid-depleted serum, the level of p53 decreased to 10% of the control and returned to near control level upon treatment for 1–24h with 1 nM E_2 . E_2 treatment of cells resulted in a steroid specific increase in p53 mRNA level which remain unchanged upon treatment with R5020 and antiestrogens. Our data suggests that the rate of T47D cell proliferation and the levels of p53 are regulated by E_2 . This effect was sensitive to pure antiestrogen ICI-164384. Furthermore, the E_2 effects appear to be ER-mediated and at the level of transcription of the p53 gene. Supported by Research Excellence Fund, Oakland Univ. and NIH-DK20893.

#1971 Development of ovarian hormone dependent mammary tumors in polyomavirus-infected female nude mice. Wirth, J.J., Haslam, S.Z., and Fluck, M.M. *Michigan State University, East Lansing, MI 48824*

Virtually nothing is known about factors which determine the development of hormone-dependent vs hormone-independent breast cancer. Previous reports from our laboratory showed that age and ovarian hormone status affect mammary tumor development in polyomavirus (Py) infected female nude mice such that mice infected at 20 and 30 weeks of age have a lower incidence and tumor latency and develop fewer tumors compared to mice infected at 3 or 6 weeks of age. Ovariectomy (ovx) decreases tumor induction in mice infected at 6, 10 and 20 weeks of age, with older mice being more profoundly affected. (Rondinelli, et al., 1995 *Oncogene* 11: 1817). The purpose of the present study was to investigate whether age at the time of infection has any influence on the hormone dependence of the tumors. 10 week old female nude mice were infected with Py and were ovx 5 weeks before mammary tumor development, 5 weeks shortly after palpable tumors were detected or left untreated. All mice were monitored for tumor development. OvX either before or after mammary tumor development lead to a reduction in tumor incidence. The tumor of one mouse ovx after tumor development completely regressed. These preliminary results indicate age and mammary gland status at the time of tumor growth directly or indirectly affect the hormone dependence of mammary tumors. Data will be presented on the histopathology and expression of viral and cellular oncogenes.

#1972 Expression of estrogen receptor variant mRNAs and determination of estrogen receptor status in human breast cancer. Huang, A., Leygue, E., Snell, L., Murphy, L.C., and Watson, P.H. *Depts. of Pathology and Biochemistry & Molecular Biology, University of Manitoba, Winnipeg, Manitoba, R3E 0W3, Canada*

Estrogen receptor (ER) status of breast cancer can be assessed by immunohistochemical assay (IHA), however we have previously observed that ER-IHA levels can be inconsistent between N-terminal and C-terminal targeted antibodies (Huang, J. Path., 1996). To address the hypothesis that this discrepancy is attributable to expression of ER variant mRNAs encoding truncated ER-like proteins we have studied 39 'IHA-consistent' and 24 'IHA-inconsistent' breast tumors by reverse-transcription-polymerase-chain-reaction (RT-PCR) to examine the expression of multiple exon-deleted (D-ER) variant mRNAs and the truncated ER clone 4 variant mRNA. ER variants D7-ER, D4-ER, D3-4-ER, and D4-7-ER were detected at similar frequencies in both groups. However, ER variants D2-3/7-ER, D2-3-4-ER ($P<0.05$) and D-3-7-ER ($P<0.01$) which encode putative short ER-like proteins that might be recognized only by an N-terminal targeted antibody, were preferentially detected in 'inconsistent' cases. ER clone 4 mRNA expression was also higher in inconsistent tumors ($P<0.001$). Further analysis showed that whereas overall prevalence of ER variant mRNAs was similar in both tumor groups, occurrence of the subset of variant mRNAs encoding putative truncated proteins was also higher in 'IHA-inconsistent' tumors ($P<0.05$). These data suggest that ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER-IHA levels determined using N- or C-terminal targeted antibodies. Supported by grants from the CBCRI, NCIC, and USAMRMC.

#1973 Two promoters in expression of estrogen receptor messenger RNA in human breast cancer. Hayashi, S.-I., Tanimoto, K., Eguchi, H., Hajiro-Nakanishi, K., Imai, K., Suga, K., and Nakachi, K. *Saitama Cancer Center Res. Inst., Saitama 362, Japan*

Estrogen receptor (ER) plays an important role in breast carcinogenesis. The human ER gene has recently been shown to transcribe two types of mRNA originating from two distinct promoters in mammary tumor cell lines encoding the same protein. We investigated which promoter is responsible for the expression of ER in human mammary tumors: the expression levels of total ER mRNA and two types of mRNAs from the different promoters were analyzed in tumor, surrounding tissue, and normal tissue obtained from patients with breast cancer.

In tumors, levels of total ER mRNA and the mRNA transcribed from a distal promoter showed remarkable correlation to the ER protein levels. In contrast, mRNA from a proximal promoter showed no correlation to the ER protein levels. Our results indicate that the enhancement of the ER mRNA expression from the distal promoter plays an essential role in the mechanisms of overexpressing ER protein in human mammary tumors. Molecular mechanisms of enhancing the distal promoter activity were further investigated, using luciferase-reporter plasmid and gel-mobility shift assays.

#1974 Uterine estrogen sulfatase plays a more important role than hepatic sulfatase in determining the sensitivity of the uterus to estrone-3-sulfate. Zhu, B.T., and Fu, J.-H. *Lab. for Cancer Research, Dept. of Chem. Biology, College of Pharmacy, Rutgers University, Piscataway, NJ 08855-076 U.S.A.*

Liver microsomes from adult female CD-1 mice, adult female Sencar mice, and adult female C3H/OuJ mice all contained <2% of the estrone-3-sulfatase activity found in liver microsomes from adult female Sprague-Dawley rats or adult female Long-Evans rats. In contrast, uterine whole homogenates from immature female CD-1 mice contained comparable levels of estrone-3-sulfatase activity (30–60% as were detected in uterine whole homogenates from immature female Sprague-Dawley rats. The ED50 values for the uterotrophic effect of estrone-3-sulfate and estrone in immature female CD-1 mice were 90 and 2 ng/g body weight, respectively, and the corresponding ED50 values in immature female Sprague-Dawley rats were 320 and 16 ng/g body weight, respectively. The body weight-adjusted uterine sensitivity of immature CD-1 mice to s.c. injected estrone-3-sulfate (calculated based on the ratio of the ED50 for estrone-3-sulfate to the ED50 for estrone) is approximately 50% of the sensitivity of immature Sprague-Dawley rats, which correlates closely with their differences in uterine estrogen sulfatase activity but not the hepatic sulfatase activity. The results of our study suggest that the estrogen sulfatase in uterus (an estrogen target organ) plays a more important role than hepatic sulfatase in mediating the hormonal action of sulfonated estrogens.

#1975 In situ produced estrogen plays more important role than circulating estradiol in tumor promotion. Yue, W., Wang, J.-P., Hamilton, C., Demers, L., and Santen, R.J. *University of Virginia, Charlottesville, VA 22908, Penn State Univ., Hershey, PA 17033*

Breast carcinomas can convert androgens to estrogens *in situ* through the enzyme, aromatase and stimulate tumor growth via an autocrine effect. However, the quantitative magnitude of autocrine versus endocrine effect has never been evaluated. In this study, we compared growth rate of aromatase transfected MCF-7 cells in ovariectomized (OVX) nude mice treated either with an aromatase substrate, androstenedione (Δ^4A , 0.1 mg/day) (autocrine model) or with E_2 delivered by silastic implants (endocrine model). The autocrine effect stimulated tumor weight to a greater extent (449 ± 70 mg) than the endocrine effect provided by the highest dose of E_2 designed to produce 10 pg/ml of plasma E_2 level (231 ± 30 mg). Because of the sensitivity limit of current available methods for measurement of mouse serum E_2 levels, uterine weight was used as an indicator of circulating E_2 content. In contrast to tumor growth, greater uterotrophic effect was observed in all E_2 treated mice than in Δ^4A treated one. Treatment with an aromatase inhibitor reduced tumor E_2 levels and inhibited tumor growth. Our results demonstrate that *in situ* aromatization substantially increases tumor E_2 levels and tumor growth rates.

#1976 High expression of the lipocalin 24p3 correlates with negative ER and PgR levels in breast cancer cells in vivo and in vitro. Chen, K.-S., Stoesz, S.P., Lindstrom, M.J., Clark, G.M., and Gould, M.N. *UW-Madison, Depts. of Human Oncology and Biostatistics, Madison, WI 53792, Medicine/Oncology, UT Health Science Center, San Antonio, TX 78284*

The lipocalin 24p3 has been previously reported to be associated with highly aggressive neu-induced rat mammary carcinomas. In order to extend this investigation to human breast cancer, we characterized 120 human breast carcinomas for HER2/neu amplification and 24p3 expression. Surprisingly, we found no correlation ($p=0.5624$). However, we did find a strong association between high 24p3 levels and ER- ($p=0.0001$) and PgR-negative status ($p=0.0004$) and high S-phase fraction ($p=0.0011$) in 250 breast cancer patients. These observations were extended to several breast cancer cell lines. Cell lines negative for ER and PgR, MDA-MB-231 and T47D:C4:2W, had high levels of 24p3, while cell lines positive for both steroid receptors, T47D:A18 and MCF-7:WS8, expressed little or no 24p3. We are currently characterizing the human and rat 5' region of the 24p3 gene to delineate mechanisms underlying these observations. Both 5' regions have been cloned and sequenced, and found to contain several consensus binding sequences for transcriptional factors, including TATA-like box, NF-1 and NF-kB binding sites, and two negative GRE/PRE elements. These results suggest that 24p3 has the potential to serve as a downstream marker of ER/PgR-negative status in breast cancer.

#1977 Induction of reversible growth arrest of immortal and neoplastic human breast epithelial cells by human chorionic gonadotropin (hCG). Mgbonye, O.P., Russo, J., and Russo, I.H. *Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA 19111, USA*

APPENDIX 37

Coutts A, **Leygue E** and Murphy LC

Mechanisms of hormone-independence in human breast cancer cells.

88th Annual Meeting, American Association for Cancer research (AACR).
San Diego, California, Abstract 1988, 1997.

Breast tumor cells had marginally detectable transcripts, benign breast cells had transcripts if detectable initiating from PEI1 primarily and PEI3. In both treatment with dexamethasone (D), phorbol ester (PDA) and cAMP increased transcripts substantially; by D using PEI4, PCA+cAMP using PEI1, and D+PCA+cAMP using PEI4 and PEI1. PEI1/PEI2/PEI2a use was not detectable in any breast samples. Therefore, in breast myofibroblasts both glucocorticoid receptor, and intracellular signalling molecule (cAMP and PDA) activation of protein kinase, mechanisms are implicated in transcriptional regulation of aromatase. (Supported by NCI RO1 CA65622)

#1984 Loss of melatonin is not a significant contributor to MCF-7 and BG-1 cell proliferation. Baldwin, W.S., Risinger, J.L., and Barrett, J.C. *National Institute of Environmental Health Sciences, RTP, NC 27709*

Epidemiological evidence suggests that electromagnetic fields (EMF) induce a variety of cancers, including breast cancer. It has been postulated that EMF induces breast cancer due to the inhibitory effects EMF has upon melatonin production. Melatonin may act through several proposed mechanisms to inhibit breast and other cancers. We examined two of these mechanisms *in vitro*. Does melatonin protect cells from oxygen radicals and does it attenuate estrogen induced cell growth? Melatonin protected MCF-7 cells from H₂O₂-induced death at 10 μ M concentrations, but not at physiological concentrations. Next, we examined melatonin's effects upon estradiol-induced proliferation in MCF-7 and BG-1 cells. Melatonin attenuated proliferation during basal growth, but not following estradiol exposure. This work was also repeated with estrogen starved cells to mimic *in vivo* conditions with no difference in results. Further work demonstrated that melatonin could not inhibit estradiol-induced G1 to S phase cell cycle transition, nor could it inhibit estradiol-induced PS2 mRNA levels. Both proliferation studies and the use of biomarkers demonstrated that melatonin does not inhibit estradiol-induced proliferation, suggesting that EMF-induced loss of melatonin is not important in the etiology of breast cancer. However melatonin homeostasis may have other effects upon estradiol production that were not addressed in this study.

#1985 Progesterins do not stimulate mammary gland proliferation in a postmenopausal animal model. Raafat, A., Li, S., and Haslam, S.Z. *Physiology Dept., Michigan State University, E. Lansing, MI 48824*

Estrogen (E) plus progesterin (P) are believed to cause maximal stimulation of mammary cell proliferation in the rodent and human mammary glands. While combined hormone replacement therapy (CHRT) with E+P is commonly prescribed for postmenopausal women, its effects on mammary gland proliferation and potential impact on breast cancer risk are poorly defined. The purpose of the present study was to determine the effect of E+P on mammary cell proliferation in a postmenopausal mouse model. We found that in postmenopausal mice, the stimulatory effect of E+P was the same as E alone. In contrast, E+P increased cell proliferation significantly more than E alone only in non-menopausal controls. These differences were also reflected in changes in mammary gland morphology, with ductal sidebranching observed only in E+P-treated non-menopausal controls. Analysis of E regulation of mammary PR revealed that PR levels could be increased only in the non-menopausal controls. The lack of mammary PR regulation by E in postmenopausal mice was tissue specific since E increased uterine PR to the same extent in both postmenopausal and control mice. Thus, low PR levels and absence of PR regulation by E likely explain the lack of increased mammary proliferation upon E+P treatment in postmenopausal mice. These results suggest that the addition of P in CHRT may not increase mammary proliferation or impact on breast cancer risk over that observed with E alone. Supported by NIH grant R01AG 13059.

#1986 Role of estrogen receptor in enhanced estrogen-induced mammary gland proliferation in a postmenopausal animal model. Raafat, A., Li, S., Bennett, J., and Haslam, S.Z. *Physiology Dept., Michigan State University, E. Lansing, MI 48824*

Conflicting epidemiological evidence on postmenopausal hormone replacement therapy (HRT) and increased breast cancer risk indicates that a direct analysis of HRT on mammary tissue is needed. Using ovariectomy-induced menopause in mice as a model, we have found that estrogen (E) caused 2-fold increased proliferation in mammary tissue over non-menopausal controls. The present studies were undertaken to determine if the enhanced response is due to increased E efficacy or potency. Dose response studies showed that increased E efficacy is the basis for the enhanced proliferation. To define the role of estrogen receptors (ER), ER concentration and cellular distribution were analyzed immunohistochemically. While there was no difference in the number of ER positive epithelial cells, there were 25% more ER positive stromal cells in postmenopausal glands. Furthermore, there was a significant (26%) reduction in E-induced ER down regulation in the epithelium of postmenopausal glands. Thus the higher ER content in stromal cells together with the greater persistence of ER in epithelial cells most likely contribute to the increased proliferation. These results suggest that in the postmenopausal gland, increased ER content and altered regulation could result in a significant proliferative response to HRT and possible increased breast cancer risk. Supported by NIH Grant R01AG13059

#1987 Activation of 17 β -estradiol and estrone by dimethyldioxirane and the transcriptional effects on DNAs with known base content and sequence. Yu, F., Bender, W., Weber, R., Ayyagari, S., Suchobrus, H., Hagshenas, L., Mallia, A., and Mallia, P. *University of Illinois Coll. of Med., Rockford, IL 61107*

Estrogens, used widely from hormone replacement therapy to cancer treatment, are themselves carcinogenic, causing uterine, liver and breast cancers. The mechanism is not understood. We found that estrone(E1), 17 β -estradiol(E2), diethylstilbestrol(DES) and tamoxifen(TAM) can be activated by the epoxide-forming oxidant, dimethyldioxirane(DMDO) and to inhibit rat liver nuclear and nucleolar RNA synthesis *in vitro*. A hypothesis is proposed suggesting epoxidation of estrogens is the underlying mechanism of carcinogenesis (Carcinogenesis 17, 1957-1961,1996). This reports the transcriptional effects of the DMDO activated E1, E2, DES and TAM on poly[d(I-C)], poly[d(A-T)], polydG-polydC, and polydC. The results show that there are differences in the degrees and specificities of inhibition. E1 and E2 show similar inhibition toward poly[d(I-C)], poly[d(A-T)], polydG-polydC and has little effect on polydC. DES has a very strong inhibition on polydC. TAM is only able to produce significant inhibition on polydG-polydC template. (Summer high school research fellows R.W., S.A., H.S., L.H., A.M., P.M.; supported by NCI CA-70466).

#1988 Mechanisms of hormone-independence in human breast cancer cells. Coutts, A.S., Leygue, E., and Murphy, L.C. *University of Manitoba, Wpg, MB, Canada R3C 2E3*

The evolution of breast cancer into an estrogen-independent growth phenotype marks the beginning of a more aggressive phase of the disease and is a major problem in the efficacy of endocrine therapies. In some cases, hormone-independence and resistance can occur due to loss of estrogen receptor (ER) expression, but at least 50% of tumors which have developed resistance to endocrine therapy remain receptor positive. T-47D5 human breast cancer cells are ER+ and estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line (T5-PRF) was developed from T-47D5 cells, by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen while still retaining expression of the ER. Transient transfection studies have been performed using an estrogen-responsive reporter gene system. In the absence of ligand T5-PRF cells have ~4 fold increased basal ER activity compared to the parent T-47D5 cells. Long range ER RT-PCR has also been performed to characterize the pattern of variant ER mRNA expression between the two cell lines and differential expression of ER mRNA variants was found. In particular, an ER variant mRNA, deleted in exons 3 and 4, was detected only in T5-PRF cells. These results suggest that defects in the ER structure and function, or activation of ER via ligand-independent mechanisms may underly hormone-independence in breast tumors.

#1989 Steroid receptors in breast cancer patients in Kuwait. Luqmani, Y.A., Temmim, L., Memon, A., Ali, M.A.A., and Parkar, A.H. *Kuwait Cancer Control Center [Y.A.L., L.T., A.M., M.A.A.A., A.H.P.], Kuwait University [Y.A.L., A.M.]*

Estrogen and progesterone receptors (ER, PR) were measured in cytoplasmic and nuclear extracts of breast cancer tissue from 799 patients, by ligand binding assay (LBA) or enzyme immunoassay (EIA). Receptor levels were much lower than widely reported in the literature. Frequency of positivity, using consensus cut off values, was lower than reported by the EORTC Group. Measurements by the two methods were statistically correlated, in terms of positivity based on criteria for clinical assessment, but concordance was poor, particularly for ER assayed in the same samples by the two methods. In cytosolic but not nuclear extracts, LBA gave higher median values for ER than EIA; for PR they were higher with EIA in both cell fractions. Correlation was excellent between receptor levels in cytosolic and nuclear extracts for both ER and PR using EIA; significantly better than with LBA. There was a correlation between ER and PR in both cytosolic and nuclear fractions particularly when analysis was by EIA. ER in the cytosolic fraction also correlated with PR in the nuclear fraction and ER in the nuclear fraction with PR in the cytosolic fraction, but only with EIA. Disagreement between the methods may be legitimately due to presence of receptor isoforms, which may have biological significance. Though presence of receptor in the cytosolic fraction is artifactual, its measurement by EIA parallels the level of receptor in the nuclear fraction.

#1990 Involvement of the transcription factor, E2F, in the biphasic response of human breast tumor cells to estradiol. Jain, P.T., and Gewirtz, D.A. *Departments of Pharmacology/Toxicology and Medicine, Medical College of Virginia, Box 230, Richmond, VA 23298*

The transcription factor, E2F, which is regulated by both Rb and p21^{waf1/cip1}, influences the G1 to S transition via its binding to the promoter regions of genes which control DNA synthesis, including *c-myc*. It is proposed that both the stimulation of breast tumor cell growth by physiological concentrations of estradiol (0.1-10 nM) and the inhibition of growth by pharmacological concentrations of estradiol (1-100 μ M) could involve regulation of *c-myc* expression through the transcription factor, E2F. Using an E2F binding site - luciferase reporter construct transiently transfected into MCF-7 breast tumor cells, we assessed the influence of estradiol on E2F activity. 100 μ M estradiol (which inhibits growth) reduced E2F activity by 45% while 10 nM estradiol (which stimulates growth) enhanced E2F activity by approximately 50%. In MCF-7 cells transiently transfected with an

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Dotzlaw H, **Leygue E**, Coutts AS , Douglas D,
Huang A, Watson PH, and Murphy LC

Steroid Hormone Receptor Variant mRNAs in Human Mammary Tissue.

3rd Internet World Congress of Biomedical Sciences. Riken, Tsukuba, Japan.
(<http://www.3iwc.riken.go.jp/CONGRESS/WELCOME.html>.) Poster
SAQ0117-AB0102, 1996.

Steroid Hormone Receptor Variant mRNAs in Human Mammary Tissue.

Dotzlaw H, Leygue E, Coutts AS, Douglas D, Huang A*, Watson PH*, Murphy LC.

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Summary.

Perturbations of the estrogen receptor (ER) and possibly the progesterone receptor (PR) signal transduction pathways are thought to play an important role in the development of breast cancer as well as its progression from hormone dependence to independence. We have identified several ER and PR variant mRNAs in both normal and neoplastic human breast tissues and have found that the frequency and level of expression of several of these variant transcripts are different in normal versus neoplastic breast tissues. Altered expression of several variants is associated with poor prognostic variables in human breast tumor biopsies and lack of endocrine sensitivity in human breast cancer cells. It is likely that the expression of altered or variant forms of either ER and PR will have an impact on ER and PR signal transduction, as well as interfere with the measurement of important markers of treatment response and prognosis in human breast cancer. Overall our data support the hypothesis that the altered expression of variant ER and PR in human breast cancer is one mechanism associated with the development and progression of human breast cancer.

Key words - estrogen receptor variant, progesterone receptor variant, breast cancer, normal breast .

Title/Authors/Summary

Introduction/Materials and Methods

Results:

1. Relationship of the Truncated Clone 4 Variant ER mRNA to some Known Prognostic Variables in Human Breast Cancer.
Expression of Clone 4 and Exon-deleted Variant ER mRNAs in Normal Human Mammary Tissues.
2. Prevalence of ER Variant mRNAs in Human Breast Cancer.
3. Expression of Novel Exon-deleted PR Variant mRNAs in Human Breast Tissues.

Conclusions/Acknowledgements/References

APPENDIX 39

Leygue E, Huang A, Murphy LC, and Watson PH

A new approach to investigate estrogen receptor variant mRNA expression
in breast tissue.

19th Annual Breast Cancer Symposium.
San Antonio, Texas, Abstract 169, 1996.

- 167 THE ACTION OF ANTIOESTROGENS IN THE BREAST CAN BE MEASURED NON-INVASIVELY USING OESTROGEN-REGULATED PROTEINS IN NIPPLE SECRETIONS. * Harding C¹, Osudenko S², L Tetlow³, Faragher B⁴, Howell A⁴, Bundred NJ¹. Depts of Surgery¹, Biochemistry² & Statistics³, Withington Hospital Dept of Medical Oncology⁴, Christie Hospital, Manchester, UK.

Tamoxifen, an antioestrogen, is being assessed as a long-term chemopreventative agent in patients at high risk of developing breast cancer. Surrogate markers of Tamoxifen action on the normal breast are needed to assess its action in individual women. Two proteins, one induced (pS2) and one inhibited (Apolipoprotein D) by oestrogen stimulation of breast cell lines, were measured in nipple secretions from normal women, and women with breast pain treated with leutenising hormone releasing hormone (LHRH) agonists or Tamoxifen, to determine if they could be used as antioestrogen markers. ApoD and pS2 were measured by radioimmunoassay and total protein by the Bradford method.

	Normal	Breast Pain	Pain after 3/12 Zol.	Pain after 3/12 Tam.
n	63	15	9	6
median pS2	9.81	27.8	6.53	4.25
range ng/mg pr	0.26-260.5	0.5-166.8	0.12-38.7	0-25.03
median ApoD	194	59.9	525.0	266.0
range ug/mg pr	12-1833.1	8.9-212.7	34-861	188.5-1773

Premenopausal secretion levels of pS2 were higher ($p < 0.02$) and ApoD lower ($p < 0.03$) than postmenopausal levels. After Zoladex and Tamoxifen treatment, secretion pS2 levels fell (both $p < 0.02$) and ApoD levels rose significantly ($p < 0.03$ and $p < 0.02$ respectively). pS2 and ApoD may prove useful intermediate markers of Tamoxifen action. Stats: Mann Whitney and Kruskal Wallis Tests.

- 168 4-HYDROXYPHENYL RETINAMIDE ENHANCES ANTI-PROLIFERATIVE EFFECT OF TAMOXIFEN ON HUMAN MAMMARY CANCER CELLS. ARAKI R, AOYAMA Y, KOGA T, YANAGA H, TAKAKI H, SHIROZU K. 1st Department of Surgery, Kurume University School of Medicine, 67 Asahimachi, Kurume, 830 Japan

It is suggested that tamoxifen (TAM) is effective in the treatment of Estrogen receptor (ER) negative cancers providing the evidence of some ER-independent mechanism, including possibility as an inducer of apoptosis. Some studies also indicate that retinoids and antiestrogens have synergistic effects on mammary carcinoma cell proliferation, via different pathways of growth inhibition. This study is designed to examine i) whether the antiproliferative effect of TAM on ER-negative and ER-positive tamoxifen resistant mammary cancer cells is enhanced in the presence of 4-Hydroxyphenyl retinamide (4-HPR), a synthetic retinoid exhibits organ specificity for breast and fatty tissue, and ii) the effect of TAM via ER-independent or ER-mediated mechanisms is implicated in the growth inhibitory action of 4-HPR in breast cancer cells *in vitro*, utilizing R-27. ER positive MCF-7 derived tamoxifen resistant mammary cancer cell line and ER negative MDA-MB-231 cell. Cells were cultured for 6 days with 96 hrs exposure of TAM and/or 4-HPR, and then cells were counted and harvested. Both cell lines exhibited inhibitory effects of TAM on anchorage dependent growth (ADG), dose-dependently. (% inhibition at $1 \mu\text{M}$ 5 μM 10 μM R-27: 15.9%, 27.3%, 50.9%, MDA 231: 0%, 17.8%, 39.2%, respectively). These effects of TAM was enhanced in the presence of non inhibitory dose of 4-HPR on both cell lines in spite of the resistance of MDA-MB231 (%inhibition 10 μM 4-HPR: 20.3%). These results may indicate that the action of 4-HPR may be implicated in the mechanism of TAM via ER-independent and/or ER-mediated pathways on ER-negative and ER-positive tamoxifen resistant mammary cancer cells, suggesting therapeutic efficacy of TAM and 4-HPR combination for ER-negative and TAM resistant breast cancers.

- 169 A NEW APPROACH TO INVESTIGATE ESTROGEN RECEPTOR VARIANT mRNA EXPRESSION IN BREAST TISSUE.

Leygue E., Huang A., Murphy LC., Watson PH. Depts of Biochemistry and Pathology, University of Manitoba, Winnipeg, Manitoba, R3E0W3, CANADA

Several exon-deleted ER variant mRNAs have been described in normal and neoplastic breast tissues. It has been suggested that changes in the level of expression of these variants could contribute to tumor progression. We developed a new approach to screen breast tissue samples for the presence of deleted ER variant mRNAs and to detect possible changes in their relative expression. This approach was based on the co-amplification of WT ER and exon-deleted ER variant cDNAs using primers annealing with proximal and distal sequences shared by the corresponding mRNAs. A competitive amplification between each of the individual transcripts occurred, that depended on their initial relative levels. Among 70 ER positive breast tumors studied using this approach, 95%, 87%, 24%, 14%, 11%, 8% and 7% expressed detectable levels of WT ER mRNA; variants deleted in exon 7 only; exon 4 only; both exons 4 and 7; both exons 3 and 4; exons 2, 3 and 4; and exons 2, 3 and 7. An exon 4-deleted ER variant was preferentially detected in tumors with lower grades ($P < 0.05$) or higher progesterone receptor levels ($P < 0.01$).

This strategy could be used to gain insights into the role of ER variants as new prognostic markers and in tumor progression.

- 170 ALTERED ESTROGEN RECEPTOR (ER) STRUCTURE ASSOCIATED WITH LOSS OF ER DNA-BINDING FUNCTION.

Lu B*, Baldwin M, Chang C-H, Scott GK, and Benz CC, University of California, San Francisco, San Francisco, California 94143.

We have shown that *in vitro* DNA-binding by 67 kDa ER is inhibited by thiol-specific ER oxidation or alkylation as well as by ionic complexing with polyamines. This inhibition of DNA-binding is associated with treatment-induced loss of ER transactivation as assessed by transient transfection of ER-positive cells with an ER-responsive reporter gene (ERE-tk-CAT). Studies with recombinantly expressed 14 kDa ER DNA-binding domain (ER-DBD) suggest that loss of DNA-binding might be due to structural changes relating to specific redox-reactive and polyamine-complexing elements within the ER-DBD. Therefore, we used circular dichroism spectrometry (CD) to detect reversible and non-reversible changes in ER-DBD secondary structure upon exposure to thiol oxidizing agents like diamide (DIA) and hydrogen peroxide (H_2O_2), a thiol alkylating agent iodoacetamide (IAA), and the natural polyamine spermine. The CD spectrum of recombinant histidine-tagged ER-DBD showed it to have significant α -helical content (characteristic minima at 208 and 221 nm), consistent with its known solution structure. Exposure to DIA or H_2O_2 under conditions that produce DTT-reversible loss of DNA-binding shifted the spectral minimum to ~200 nm, characteristic of a predominantly random coil structure; ER-DBD α -helical content was fully restored by subsequent treatment with excess DTT. IAA inhibition of DNA-binding was associated with an identical loss of ER-DBD secondary structure which could not be restored by DTT. Lastly, high concentrations of spermine produced no significant change in ER-DBD α -helical content, suggesting higher order structural changes associated with polyamine-induced loss of DNA-binding. These findings support the possibility that loss of ER DNA-binding, as observed in ~40% of human breast tumors, results from changes in ER structure including reversible redox-mediated loss of α -helical content and non-reversible loss of secondary or higher order (e.g. tertiary/quaternary) ER structure.

APPENDIX 40

Hiller T, **Leygue E**, Murphy LC, and Watson PH

Quantification of estrogen receptor clone 4 variant in microdissected normal and neoplastic human breast tissues.

87th Annual Meeting, American Association for Cancer research (AACR).
Washington D. C., Abstract 1646, 1996.

#1643 Sunday, April 21, 1996, 8:00-12:00, Room 30
Estradiol-17 β (E₂)-regulated expression of protein tyrosine phosphatase gamma (PTP- γ) gene in cultured human breast and breast cancer cells. Zheng, J.¹, Zhang, Y.¹, Sugimoto, Y.¹, Mulla, Z.¹, Canatan, H.¹, Dayton, M.A.², Govindan, M.V.³, Farrar, W.B.¹, Brueggemeier, R. W.¹, and Lin, Y.C.¹ ¹The Ohio State Univ., Columbus, Ohio 43210, ²Louisiana State Univ., Shreveport, LA 71130, ³Laval Univ., Quebec, Canada.
PTP- γ is a potential tumor suppressor gene in human kidney and lung cancers. Our previous results have also shown that PTP- γ mRNA is expressed in both primary cultured human breast cells and breast cancer cell lines by RT-PCR. By RNase protection assay, we found that PTP- γ gene expression level was lower in human breast cancer cells than that in the normal breast cells. We also demonstrated that PTP- γ mRNA expression was inhibited by E₂ dose-dependently in primary cultured normal breast cells. After the cells were treated with 20nM of E₂ for 24 hours, PTP- γ mRNA expression was significantly inhibited in both primary cultured cancerous and non-cancerous cells from breast cancer patients, as well as in estrogen receptor (ER)-positive MCF-7 cell line by 50%, 85%, and 66%, respectively. However, the expression level did not change in the ER-negative MDA-MB-231 cell line. PTP- γ mRNA expression was significantly inhibited (by 94%) in ER-transfected MDA-MB-231 transfectants which was transfected with an ER expression plasmid. On the other hand, 1 μ M of progesterone significantly stimulated PTP- γ mRNA expression by 7-fold. Our results showed that estrogen significantly inhibited PTP- γ gene expression in cultured human breast cells and this inhibition is mediated by ER, while progesterone significantly stimulated it. Our results is the first to suggest that PTP- γ is a potential estrogen-regulated tumor suppressor gene in human breast cancer and may play an important role during neoplastic processes in the human breast. (Supported by NIH grants DK45916, CA58003 and CA66193.)

#1644 Monday, April 22, 1996, 1:00-5:00, Poster Section 9
Leukemia inhibitory factor (LIF) and its receptor (LIFR) in breast cancer: a potential autocrine/paracrine growth regulatory mechanism. Dhangra, K., Sahin, A., Emami, K., Estrov, Z. M.D. *Anderson Cancer Ctr, Houston, TX.*
Human breast tumors show a high propensity to metastasize to the bone/bone marrow. This may be due to the growth stimulatory effect of hematopoietic cytokines on breast tumor cells. We have recently shown that cultured human breast cancer cells express LIFR and can be stimulated to grow by LIF (which is known to be constitutively produced by bone marrow stromal cells) (J. Interferon Cytokine Res., 15:905, 1995). To investigate the *in vivo* relevance of these observations, we developed an immunohistochemical staining method for LIFR using a monoclonal antibody M1 (kindly provided by Bettina Thoma, Immunex, Inc.). LIFR expression was detected in 79% (38/48) specimens. In 29 specimens, the expression was detected at a high frequency (50-75% positive cells-4 specimens; 75-100% positive cells-25 specimens). Interestingly, the majority (80%) of these specimens also showed positive LIF immunostaining (using a monoclonal antibody D62.3.2, kindly provided by K. Jin Kim, Genentech, Inc.). LIFR positivity was higher in well-differentiated tumors (p = 0.04) but expression of LIF/LIFR did not correlate with other conventional prognostic features. These findings support a potential role for LIF/LIFR in autocrine/paracrine growth regulation of breast cancer.

#1645 Monday, April 22, 1996, 1:00-5:00, Poster Section 9
Expression of tumor markers during breast tumor progression. Wani, G., Noyes, I., Milo, G.E., and D'Ambrosio, S.M., *Depts. Radiology & Medical Biochemistry, The Ohio State University, Columbus, OH 43210.*
Surgical specimens of breast tubular ductal carcinomas, and invasive ductal carcinomas, were grafted onto 3-4 wk old female nude mice. Fresh tumor tissue and progressively growing tumors were harvested every two wk and analyzed immunohistochemically. The expression of molecular biomarkers (estrogen receptor, p53, CerbB-2, and cyclin D1) associated with the aggressive nature of the tumor were characterized as a function of surrogate tumor progression and compared to the fresh tumor. The data indicates that: (1) in tubular ductal carcinomas the diversity of cellular morphology is conserved in the xenograft. (2) A strong specific staining of ductal cells compared to other structures in both the tumor and progressively growing surrogate tumor was observed for the estrogen receptor, cyclin D1, and CerbB-2. (3) A diffused and heterogeneous staining was observed for p53. These spatial areas of molecular biomarker expression observed in both the fresh and surrogate tumors were not apparent in normal breast tissue. These data support the tenet that the diversity of cellular morphology and differential spatial expression of molecular biomarkers is consistent with the proposal that the heterogeneous cellular matrix of the breast tumor indicates that there are diverse phenotypes present during the different stages of the progression in both the tumor and surrogate tumor. (Supported by NCI grant P20-66193.)

#1646 Monday, April 22, 1996, 1:00-5:00, Poster Section 9
Quantitation of estrogen receptor clone 4 mRNA variant in microdissected normal and neoplastic human breast tissues. Hiller, T.¹, Leygue, E.², Murphy, L.², Watson, H.¹, *Departments of Pathology¹ and Biochemistry & Molecular Biology², University of Manitoba, Winnipeg, Canada. R3E 0W3.*
Estrogen receptor (ER) mRNA variants may play a role in progression in human breast cancer and we have also found that the expression of ER deletion 5 variant

relative to wild type ER (ER wt) can differ between normal and neoplastic breast tissue (Leygue et al, JNCI, in press). To pursue the role of 'truncated' and 'deleted' ER mRNA variants in the development of breast cancer further we have developed a novel approach to facilitate microdissection and RNA extraction from small pathologically defined regions within frozen breast tissue sections. Histologically defined areas less than 2 mm² can be microdissected to provide an average yield of 1.0 μ g (+/- 0.5 μ g) of total RNA from 5 serial 20 μ m sections. We have also developed a specific Triple-Primer RT-PCR assay to assess the expression of the truncated ER clone 4 mRNA variant relative to the wt ER based on internal competition between 3 primers. A preliminary study of unmatched samples showed a significant increase in the level of ER clone 4 in tumors relative to normal (ER clone 4/ER wt, median tumor level = 107.5%, n = 10; median normal level = 82.5%, n = 8; p = 0.03). We have now begun to extend our analysis of ER clone 4 and ER deletion variant expression to regions of normal, hyperplastic, in-situ and invasive components within single tumor tissue sections. Supported by Canadian Breast Cancer Initiative and USAMRDC.

#1647 Monday, April 22, 1996, 1:00-5:00, Poster Section 9
Quantitative reverse transcription polymerase chain reaction analysis of c-myc expression in breast cancer. Singh R., & Watson P.H. *Dept of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada. R3E 0W3.*
Alteration of c-myc gene expression has been implicated in the progression of breast cancer and the transition to hormone independent growth. As a first step to determine the clinical relevance, we have studied the effect of specimen collection time of tumors on the level of c-myc expression. We have developed competitive RT-PCR assays using synthetic cRNA standards to quantitate c-myc mRNA and estrogen receptor (ER) mRNA extracted from small microdissected breast tumor samples. The c-myc assay could accurately distinguish a minimal 2-fold difference by comparison with Northern blot and the ER assay could distinguish a 100 fold difference in ER level in mixtures of ER+ve and ER-ve breast cell lines. We then assessed the relatively unstable c-myc mRNA and the stable ER mRNA in multiple homogeneous samples collected from 10 breast tumor surgical specimens and stored on ice for 0, 3, 6 and 24 hours prior to freezing and subsequent RNA extraction. The levels of c-myc declined over 24 hours in each case (mean of 74% of the level at time 0, sd 77%, max 31%, min 93%, n = 10) while ER levels showed smaller reductions (mean 95%, max 91%, min 97%, n = 4). Small differences in the rate of c-myc decline were independent of the tumor pathology assessed in adjacent tissue blocks. We conclude that knowledge of the time of collection of surgical specimens may be an important parameter to assess the role of alteration of c-myc mRNA gene expression (supported the Canadian Breast Cancer Initiative).

#1648 Monday, April 22, 1996, 1:00-5:00, Poster Section 9
Identification of Genes Differentially Expressed in MCF-7 Breast Carcinoma Cells Upon Treatment with 17 β -Estradiol. Go, V. and Pogo, B. G.T. *Mount Sinai School of Medicine, New York, NY 10029*
The human breast carcinoma cell line MCF-7 produces tumors with an increased growth rate and metastatic potential in athymic mice upon the co-introduction of 17 β -estradiol (Shafie and Liotta, Cancer Letters 11:81-87, 1980). Furthermore, the presence of an estrogen-insulin axis adds to the complexity of unraveling the molecular mechanisms behind estradiol induced mammary tumor growth.
In order to determine the genes involved in increased tumor growth and metastasis as a result of estradiol induction, we have extracted total RNA from MCF-7 cells supplemented with insulin along with or without 17 β -estradiol treatment. The differential display technique was implemented resulting in 91 possible differentially expressed cDNA fragments of which 21 were found to be real. Northern analysis was used to reconfirm the differences, and the differentially expressed fragments were cloned and sequenced revealing unique estradiol induced sequences.

#1649 Monday, April 22, 1996, 1:00-5:00, Poster Section 9
Bifunctional hormonal/genetic pathways to breast cancer. Devra Lee Davis*, H. Leon Bradlow*, Jack Fishman*, Michael Osborne*, Nitin Telang*. **World Resources Institute, 1709 New York Avenue N.W., Washington, DC 20006, *Strang Cornell Cancer Research Laboratory, Cornell University Medical College, New York, NY 10021*
Inherited germ line mutations, such as loss of BRCA1 or AT, appear to account for less than 5% of all breast cancer. Postnatal perturbations in the genome appear to be involved in most cases of the disease. Cumulative lifetime exposure to bioavailable estradiol links most known risk factors for breast cancer, excepting radiation. Based on a series of recent experimental and epidemiologic findings, we hypothesize that endogenous or exogenous hormones exert bi-functional biologic effects on hormonal and/or genetic paths to breast cancer. Depending on the periods and extent of exposure, the operative toxicologic consequences can be chiefly genetic, epigenetic, or hormonal. Prenatal, pre-pubescent, or adolescent exposures to some xenohormones can work principally through genetic or epigenetic mechanisms chemically imprinting developing cells and modify DNA structure or function, affecting replication, repair processes, and gene regulation. Functional damage can affect such processes as phosphorylation of p53, jun, fos, RB and phosphatidylinositol-3-kinase. This impedes critical cell repair systems, hindering recognition of damaged cells and allowing the accumulation of

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CURRICULUM VITAE

Etienne LEYGUE, Ph. D.

French citizenship, 35 years old

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Present Position

1995... Postdoctoral Fellow

Dept of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Canada

Study of variant forms of steroid receptors in normal and neoplastic human breast tissues and identification of genes involved in tumor progression.

Academic degrees

1994 Ph.D., With distinction

University Pierre and Marie Curie, Paris, France

Expression and estrogen-dependence of c-myc proto-oncogene in normal human breast epithelial cells in culture.

1990 DEA (predoctoral Degree), With distinction

University Pierre and Marie Curie, Paris, France

1989 Master's in Biology, With distinction

University Paul Sabatier, Toulouse, France

Teaching experience and qualification

1998... Qualified to teach Cell Biology and Molecular Biology in French Universities

1992-1994 Molecular Biology

University Pierre and Marie Curie, Paris, France

Teaching Molecular Biology and Cellular Biology to First year Graduate Students

1988-1989 Biology

College "La Rouatiere", Castelnaudary, France

Teaching Plant Biology to High School Students

Honours and Awards

1996-1999 US Army Medical Research and Materiel Command Postdoctoral Fellowship

1995 Faculty Fund Postdoctoral Fellowship, *University of Manitoba, Canada*

1994 Grant from "Ligue Nationale Francaise Contre le Cancer", *France*

1990-1994 4 Grants "INSERM-CFB": 8 months training periods in the Laboratory of Molecular Oncology, *University of Liège, Belgium*

1990-1993 Grant from Minister of Research and Technology, *Paris, France*

1990 Head of the class of 1990, DEA Reproductive Physiology

And....

Lieutenant in the French Army Reserves, Instructor-Paratroopers Certificate, Commando Certificate, Award of the National Defense Bronze Medal, Long-distance runner (Marathon), Kayak, Rugby...

Publications and Communications (August 1999)

Articles and reviews published or in press in peer reviewed journals

- 30 Coutts, A. S., **Leygue, E.**, and Murphy, L. C., Variant estrogen receptor alpha messenger RNA in hormone independent human breast cancer cells. *J Mol Endocrinol*, In press.
- 29 Lu, B., Dotzlaw, H., **Leygue, E.**, Murphy, L.J., Watson, P. H., and Murphy, L. C. Estrogen receptor alpha mRNA variants in murine and human tissues. *Mol Cell Endocrinol*, In press.
- 28 **Leygue, E.**, Dotzlaw, H., Watson, P. H., and Murphy, L. C. Expression of the steroid receptor RNA activator (SRA) in human breast tumors. *Cancer Res*, In press.
- 27 **Leygue, E.**, Snell, L., Dotzlaw, H., Hole, K., Hiller-Hitchcock, T., Murphy, L. C., and Watson, P. H. Lumican and decorin are differentially expressed in human breast carcinoma. *J Pathol*, In press.
- 26 Huang, A., **Leygue, E.**, Dotzlaw, H., Murphy, L. C., and Watson, P. H. Estrogen receptor mRNA variants influence the determination of ER status in human breast cancer. *Breast Cancer Res Treat*, In press.
- 25 Dotzlaw, H., **Leygue, E.**, Watson, P. H., and Murphy, L. C. The human orphan receptor PXR is expressed in both normal and neoplastic breast tissue. *Clin Cancer Res*, In press.
- 24 **Leygue, E.**, Dotzlaw, H., Lu, B., Watson, P. H. and Murphy, L. C. Estrogen receptor beta: a review. *Cur Top Steroid Res*, In press.
- 23 **Leygue, E.**, Snell, L., Dotzlaw, H., Hole, K., Troup, S., Hiller-Hitchcock, T., Murphy, L. C., and Watson, P. H. Mammaglobin, a potential marker of breast cancer micrometastasis. *J Pathol*, 189: 28-33, 1999.
- 22 **Leygue, E.**, Dotzlaw, H., Watson, P. H., and Murphy, L. C. Altered expression of exon 6 deleted progesterone receptor variant mRNA between normal human breast and breast tumor tissues. *Brit J Cancer*, 80: 379-382, 1999.
- 21 **Leygue, E.**, Dotzlaw, H., Watson, P. H., and Murphy, L. C. Expression of estrogen receptor beta 1, beta 2 and beta 5 mRNAs in human breast tissue. *Cancer Res*, 59:1175-1179, 1999.
- 20 Dotzlaw, H., **Leygue, E.**, Watson, P. H., and Murphy, L. C. Estrogen receptor-b mRNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. *Cancer Res*, 59:529-532, 1999.
- 19 **Leygue, E.**, Hall, R., Dotzlaw, H., Watson, P. H., and Murphy, L. C. Estrogen receptor-a variant mRNAs expression in primary human breast tumors and matched lymph node metastases. *Brit J Cancer*, 79:978-983, 1999.
- 18 **Leygue, E.**, Dotzlaw, H., Lu, B., Glor, C., Watson, P. H. and Murphy, L. C. Estrogen receptor beta: mine is longer than yours? *J Clin Endocrinol Metab*, 83:3754-3755, 1998.
- 17 **Leygue, E.**, Dotzlaw, H., Watson, P. H., and Murphy, L. C. Altered estrogen receptor alpha and beta expression during human breast tumorigenesis. *Cancer Res*, 58:3197-3201, 1998.
- 16 Murphy, L. C., Dotzlaw, H., **Leygue, E.**, Coutts, A., and Watson, P. H. The pathophysiological role of estrogen receptor variants in human breast. *J Steroid Biochem Mol Biol*, 65:175-180, 1998.
- 15 Lu, B., **Leygue, E.**, Dotzlaw, H., Murphy, L.J., Murphy, L. C., and Watson, P. H. Estrogen receptor beta mRNA variants in human and murine tissues. *Mol Cell Endocrinol*, 138:199-203, 1998.
- 14 Watson, P. H., **Leygue, E.**, and Murphy, L. C. Molecules in focus: Psoriasis (S100A7). *Int J Biochem Cell Biol*, 30: 567-571, 1998.
- 13 **Leygue, E.**, Snell, L., Dotzlaw, H., Hole, K., Hiller-Hitchcock, T., Roughley, P., Murphy, L. C., and Watson, P. H. Expression of lumican in human breast carcinoma. *Cancer Res*, 58: 1348-1352, 1998.

- 12 Murphy, L. C., Dotzlaw, H., **Leygue, E.**, Douglas, D., Coutts, A., and Watson, P. H. Estrogen receptor variants and mutations. *J Steroid Biochem Mol Biol*, 62: 363-372, 1997.
- 11 Murphy, L. C., **Leygue, E.**, Dotzlaw, H., Douglas, D., Coutts, A., and Watson, P. H. Estrogen receptor variants and mutations in human breast cancer. *Ann Med*, 29: 221-224, 1997.
- 10 Dotzlaw, H., **Leygue, E.**, Watson, P. H., and Murphy, L. C. Expression of estrogen receptor-beta in human breast tumors. *J Clin Endocrinol Metab*, 82: 2371-2374, 1997.
- 9 Huang, A., **Leygue, E.**, Snell, L., Murphy, L. C., and Watson, P. H. Expression of estrogen receptor variant mRNAs and determination of estrogen receptor status in human breast cancer. *Am J Pathol*, 150: 1827-1833, 1997.
- 8 **Leygue, E.**, Snell, L., Hiller, T., Dotzlaw, H., Hole, K., Murphy, L. C., and Watson, P. H. Differential expression of psoriasin mRNA between in-situ and invasive human breast carcinoma. *Cancer Res*, 56: 4606-4609, 1996.
- 7 **Leygue, E.**, Huang, A., Murphy, L. C., and Watson, P. H. Prevalence of estrogen receptor variant mRNAs in human breast cancer. *Cancer Res*, 56: 4324-4327, 1996.
- 6 **Leygue, E.**, Dotzlaw, H., Watson, P. H., and Murphy, L. C. Identification of novel exon-deleted progesterone receptor variant mRNAs in human breast tissue. *Biochem Biophys Res Commun*, 228: 63-68, 1996.
- 5 **Leygue, E.**, Watson, P. H., and Murphy, L. C. Identification of differentially expressed genes using minute amount of RNA. *Biotechniques*, 21: 1008-1012, 1996.
- 4 **Leygue, E.**, Murphy, L., Kuttann, F., and Watson, P. H. Triple primer polymerase chain reaction. A new way to quantify truncated mRNA expression. *Am J Pathol*, 148: 1097-1103, 1996.
- 3 **Leygue, E.**, Watson, P. H., and Murphy, L. C. Estrogen receptor variants in normal human mammary tissue. *J Natl Cancer Inst*, 88: 284-290, 1996.
- 2 Gompel, A., Martin, A., Simon, P., Schoevaert, D., Plu-Bureau, G., Hugol, D., Audouin, J., **Leygue, E.**, Truc, J. B., and Poitout, P. Epidermal growth factor receptor and c-erbB-2 expression in normal breast tissue during the menstrual cycle. *Breast Cancer Res Treat*, 38: 227-235, 1996.
- 1 **Leygue, E.**, Gol-Winkler, R., Gompel, A., Louis-Sylvestre, C., Soquet, L., Staub, S., Kuttann, F., and Mauvais-Jarvis, P. Estradiol stimulates c-myc proto-oncogene expression in normal human breast epithelial cells in culture. *J Steroid Biochem Mol Biol*, 52: 299-305, 1995.

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- 5 **Leygue, E.**, Dotzlaw, H., Simon, S.L.R., Watson, P. H., and Murphy, L. C. Increased expression of the steroid receptor RNA activator during human breast tumorigenesis. Submitted, *Cancer Res*.
- 4 **Leygue, E.**, Huang, A., Dotzlaw, H., Stickles, S., Watson, P. H., Khan, S., and Murphy, L. C. Expression of estrogen receptor alpha variant mRNAs in normal human breast tissue of women with and without breast cancer. Submitted, *Clin Cancer Res*.
- 3 Al-Haddad, S., Zhang, Z., **Leygue, E.**, Snell, L., Huang, A., Niu, Y., Hiller-Hitchcock, T., Hole, K., Murphy, L. C., and Watson, P. H. Psoriasin (S100A7) expression and invasive breast cancer. Submitted, *Am J Pathol*.
- 2 Lu, B., **Leygue, E.**, Dotzlaw, H., Murphy, L.J., and Murphy, L. C. Functional characteristics of a novel estrogen receptor beta isoform, estrogen receptor beta 2. Submitted, *Mol Endocrinol*.
- 1 **Leygue, E.**, Dotzlaw, H., Watson, P. H., and Murphy, L. C. Altered expression of estrogen receptor alpha variant mRNAs between adjacent normal breast and breast tumor tissues. Submitted, *Breast Cancer Res*.

Book chapters

- 5 Murphy, L. C., **Leygue, E.**, Dotzlaw, H., Coutts, A. S., Lu, B., Huang, A., Watson, P. H. Multiple facets of the estrogen receptor in human breast cancer. In *Endocrine Oncology*. S. Ethier ed., Humana Press, In press.
- 4 Watson, P. H., Hiller, T., Snell, L., Hole, K., Singh, R., Murphy, L. C., **Leygue, E.**, Dotzlaw, H., Kossakowska, A., and Kulakowski, A. Update to: Microdissection RT-PCR Analysis of gene expression in pathologically defined frozen tissue sections. In "The PCR techniques: RT-PCR", P. Siebert ed., Eaton Publ., Maryland, pp 28-29, 1997.
- 3 Kuttenn, F., Malet, C., **Leygue, E.**, Gompel, A., Gol, R., Baudot, N., Louis-Sylvestre, C., Soquet, L., and Mauvais-Jarvis, P. Antiestrogen action of progestogens on human breast cells. In "The modern management of the menopause", Parthenon Publ., New York, pp 419-433, 1993.
- 2 Kuttenn, F., Gompel, A., Malet, C., **Leygue, E.**, Baudot, N., Plu-Bureau, G., Thalabard, J. C., and Mauvais-Jarvis, P. The influence of steroid hormones and antihormones on the growth and differentiation of normal human breast cells in culture. In "Hormonal Carcinogenesis", J. Li, S. Nandi and S.A. Li eds., Springer-Verlag Publ., New York, pp 145-156, 1992.
- 1 Malet, C., Gompel, A., **Leygue, E.**, Baudot, N., Kuttenn, F., and Mauvais-Jarvis, P. Action of estradiol and its antagonists-progestins and antiestrogens on normal human breast cells in culture. Rationale for hormonal treatment of Benign Breast Disease. In "Benign Breast Disease of Breast and Genital Tract", F. Dambrozio, D. Amy and E. Durante eds., Eur. Soc. Breast Echography Publ., Ferrara (Italia), pp 9-20, 1992.

Abstracts and oral communications

- 33 Lu, B., Dotzlaw, **Leygue, E.**, Watson, P. H., and Murphy, L. C. Estrogen receptor- β (ER- β) variants in human breast tissues. Breast Cancer Research: Reasons for Hope. Canadian Breast Cancer Research Initiative National Scientific Conference. Toronto, Ontario, Abstract number to be announced, 1999.
- 32 Dotzlaw, **Leygue, E.**, Huang, A., Snell, L., Watson, P. H., and Murphy, L. C. Estrogen receptor (ER) variants in human breast cancer. Breast Cancer Research: Reasons for Hope. Canadian Breast Cancer Research Initiative National Scientific Conference. Toronto, Ontario, Abstract number to be announced, 1999.
- 31 Lu B., **Leygue, E.**, Dotzlaw, and Murphy, L. C. Putative function of a novel mouse estrogen receptor-beta isoform, estrogen receptor-beta 2. 81th Annual Meeting of the Endocrine Society. San Diego, California, Abstract P1-225, 1999.
- 30 **Leygue, E.**, Dotzlaw, H., Watson, P. H., and Murphy, L. C. Expression of estrogen receptor beta 1, beta 2 and beta 5 mRNAs in human breast tissue. 81th Annual Meeting of the Endocrine Society. San Diego, California, Abstract P3-265, 1999.
- 29 Al-Haddad, S., Yuan, Z., **Leygue, E.**, Huang, A., Hole, K., Snell, L., Leygue, C., Murphy, L. C., and Watson, P. H. Psoriasin (S100A7) expression in invasive breast cancer. 90th Annual Meeting, American Association for Cancer research (AACR). Philadelphia, Pennsylvania, Abstract 3719, 1999.
- 28 Dotzlaw, H., **Leygue, E.**, Watson, P. H., and Murphy, L. C. Relationship of estrogen receptor beta (ER-b) mRNA expression to markers of endocrine therapy sensitivity in human breast cancer. 90th Annual Meeting, American Association for Cancer research (AACR). Philadelphia, Pennsylvania, Abstract 2516, 1999.
- 27 Dotzlaw, H., **Leygue, E.**, Watson, P. H., and Murphy, L. C. The human orphan receptor PXR mRNA is expressed in both normal and neoplastic breast tissue. IBC 2nd International Symposium on Nuclear Hormone Receptor Drug discovery. Scottsdale, Arizona, Abstract 3, 1999.

- 26 **Leygue, E.,** Dotzlaw, H., Watson, P. H., and Murphy, L. C. Altered expression of estrogen receptor alpha variant mRNAs between adjacent normal breast and breast tumor tissues. Inabis 98. 5th Internet World Congress for Biomedical Sciences. <http://www.mcmaster.ca/inabis98/index.html>. Abstract 0756, 1998.
- 25 **Leygue, E.,** Snell, L., Dotzlaw, H., Hole, K., Hiller-Hitchcock, T., Murphy, L. C., Roughley, P. J., and Watson, P. H. Lumican and decorin are differentially expressed in human breast carcinoma. 21th Annual Breast Cancer Symposium. San Antonio, Texas, Abstract 180, 1998.
- 24 **Leygue, E.,** Hall, R., Dotzlaw, H., Watson, P. H., and Murphy, L. C. Conserved patterns of estrogen receptor-a variant mRNA expression in primary human breast tumors and matched lymph node metastases. 21th Annual Breast Cancer Symposium. San Antonio, Texas, Abstract 564, 1998.
- 23 Dotzlaw, H., **Leygue, E.,** Watson, P. H., and Murphy, L. C. Expression of estrogen receptor-a (ER-a) and estrogen receptor-beta (ER-b) mRNA in primary human breast tumors and matched normal human breast tissue samples. 10th International Congress of Hormonal Steroids. Quebec, Quebec, Abstract 63, 1998.
- 22 Lu, B., **Leygue, E.,** Dotzlaw, H., Watson, P. H., Murphy, L. J., and Murphy, L. C. Estrogen receptor alpha and beta (ER-a and ER-b) mRNA variants in human and murine tissues: species specific differences. 80th Annual Meeting of the Endocrine Society. New Orleans, Louisiana, Abstract P1-574, 1998.
- 21 Murphy, L. C., **Leygue, E.,** Dotzlaw, H., Lu, B., Coutts, A., Huang, A., and Watson, P. H. Multiple facets of the estrogen receptor (ER) in breast cancer. Breast and prostate cancer, Keystone Symposium. Copper Mountain, Colorado, 1998.
- 20 **Leygue, E.,** Dotzlaw, H., Hare, H., Watson, P. H., and Murphy, L. C. Expression of estrogen receptor-beta (ER-b) variant mRNAs in human breast tumors. 20th Annual Breast Cancer Symposium. San Antonio, Texas, Abstract 175, 1997.
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